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(71) Applicant: ONCOGEN LIMITED PARTNERSHIP [US/US]; 3005 First Avenue, Seattle, WA 98121 (US).

(72) Inventors: HAFFAR, Omar, K. ; 1212 Fifth Avenue North, Apt. 302, Seattle, WA 98109 (US). HU, Shiu-Lok ; 14128 175th Avenue, Redmond, WA 98052 (US). SENEAR, Allen, W. ; 6225 Woodlawn Avenue, Seattle, WA 98103 (US). TRAVIS, Bruce, M. ; 6029 29th Avenue N.E., Seattle, WA 98115 (US).

(74) Agent: MISROCK, S., Leslie; Pennie & Edmonds, 1155 Avenue of the Americas, New York, NY 10036 (US).

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(54) Title: NON-REPLICATING RECOMBINANT-MADE RETROVIRAL PARTICLES USED AS ANTIVIRAL AGENTS AND IMMUNOGENS

(57) Abstract

Nonreplicating recombinant-made retroviral particles having structural, morphological and immunological characteristics very similar to those of native human retroviruses are described. The method of the invention involves coexpression of mature retroviral core and envelope structural proteins in mammalian host cells such that the expressed retroviral proteins associate into assembled budding retroviral particles. In a particular embodiment of the invention, nonreplicating recombinant-made HIV-1 particles are produced by coinfecting mammalian host cells with a recombinant vaccinia virus carrying the Human Immunodeficiency Virus Type 1 (HIV-1) gag and protease genes and a recombinant vaccinia virus carrying the HIV-1 env gene. These nonreplicating recombinant-made HIV-1 particles have immunological and morphological characteristics closely resembling those of native HIV-1, are able to block the infectivity of live HIV *in vitro*, and are highly immunogenic *in vivo*. The recombinant-made HIV-1 particles of the invention may be used as anti-viral agents and as immunogens in vaccine formulations effective at inhibiting or preventing infection by HIV and/or the development of the Acquired Immunodeficiency Syndrome.

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NON-REPLICATING RECOMBINANT-MADE RETROVIRAL PARTICLES USED AS ANTIVIRAL AGENTS AND IMMUNOGENS

1. INTRODUCTION

The present invention is directed to noninfectious recombinant-made retroviral particles, to *in vitro* systems by which such particles can be generated, and to their use as anti-viral agents and as immunogens for prophylaxis and therapy against human retroviruses such as the human immunodeficiency virus (HIV). Recombinant-made HIV particles of the invention incorporate correctly processed HIV core and envelope proteins and are morphologically and immunologically very similar to native HIV. Yet, since the recombinant-made HIV particles of the invention do not contain all elements of the HIV genome necessary for viral replication, they are non-infectious.

2. BACKGROUND OF THE INVENTION

Two types of human retroviruses have been identified, leukemia viruses and AIDS or AIDS-related viruses. The primary targets of the human retroviruses are T lymphocytes and cells of the central nervous system. All human retroviruses are transmitted by intimate contact, blood contamination, and infection *in utero* or after birth by milk. It is likely that all human retroviruses orginated in Africa and that they encountered the human species via interspecies infection, possibly from African Green Monkeys or a related species. The human retroviruses first discovered, Human T Lymphotropic Virus Type 1 (HTLV-I) and Human T Lymphotropic Virus Type II (HTLV-II), have a preferential tropism for T4 cells and some T8 cells, share significant sequence homology, and are mainly associated with T cell leukemias and lymphomas. The other group of human retroviruses, generally called Human Immunodeficiency Viruses (HIV), is discussed in greater detail below. There are two major differences between the two types of human

retroviruses: (1) there is substantial genomic variability among various HIV isolates, whereas the genomes of HTLV-I and HTLV-II are stable; and (2) HIV entered human populations much more recently than HTLV-I or HTLV-II.

2.1. THE HUMAN IMMUNODEFICIENCY VIRUS AND AIDS

The human immunodeficiency virus (HIV) is a cytopathic retrovirus and the causative agent of the acquired immunodeficiency syndrome (AIDS). Two forms of HIV have now been identified. The prototype virus, HIV-1, previously termed lymphadenopathy-associated virus (LAV) and human T lymphotropic virus type III (HTLV-III), is responsible for the vast majority of reported AIDS cases worldwide. Another retrovirus, HIV-2, has been isolated primarily from West African patients with AIDS and is pathogenically related to HIV-1. On the genetic level, HIV-2 is actually more closely related to the simian immunodeficiency virus (SIV), a retrovirus infecting monkeys.

As of May 31, 1989, over 97,000 cases of AIDS had been reported in the United States alone, and over half of those people have already died. As many as three million persons in this country may be asymptomatic carriers of HIV and are capable of transmitting the virus. It has been estimated that 270,000 cases of AIDS will have occurred in the United States by 1991 (U.S. Public Health Service, 1986, Public Health Rep. 101: 341). The mortality rate from AIDS is disturbingly high, exceeding 80% within three years of diagnosis and possibly reaching 100% over a longer period.

Worldwide, the AIDS epidemic may involve some five to ten million presently infected persons. Particularly troublesome are statistics from the African continent where millions of individuals are believed infected with HIV, deaths range in the hundreds of thousands, and heterosexual transmission predominates. To date, there is neither a

known cure for AIDS nor an effective vaccine against HIV infection.

2.2. PATHOGENESIS OF HIV INFECTION

HIV is a member of the nontransforming, cytopathic lentivirus family of retroviruses. HIV causes a typically fatal disease characterized by severe immunodeficiency or neurodegenerative disease, or both. The primary basis for HIV induced immunosuppression is the depletion of the helper/inducer subset of T lymphocytes expressing the CD4 molecule (T4 or $CD4^+$ cells), which serves as the high affinity cell surface receptor for the virus. T4 lymphocytes are involved directly or indirectly in the induction of nearly every immunologic function in the body, and their depletion results in susceptibility to a wide range of opportunistic infections and neoplasms.

In addition to the T4 lymphocyte, other cells expressing the CD4 molecule are targets of HIV infection, especially monocyte-macrophages and certain neurons and glial cells of the brain. HIV infection also results in serious B cell abnormalities including polyclonal activation, hypergammaglobulinemia, elevated levels of circulating immune complexes, and autoantibodies. A decreased number of functional natural killer (NK) cells have also been observed in AIDS patients.

Infection of $CD4^+$ cells is initiated by the interaction of the CD4 molecule with the major HIV envelope glycoprotein gp120, an event which is followed by internalization and uncoating of the virion, transcription of genomic RNA to DNA by virus-encoded reverse transcriptase, and integration of the resulting proviral DNA into host cell chromosomal DNA. Also, unintegrated proviral DNA accumulates in large amounts within infected cells and is probably a significant factor in HIV cytopathicity (Shaw et al., 1984, Science 226: 1165).

During replication, mRNA transcripts of integrated proviral DNA are translated into HIV proteins. These proteins are then processed and assembled along with HIV genomic RNA. Mature virions bud from the surface of infected T-lymphocytes and bud internally in macrophages, incorporating host cell membrane lipid to form virion envelope.

Although HIV may remain dormant for some time after infection, when active replication of virus occurs, the host $CD4^+$ cell is usually killed. The precise mechanism by which HIV exerts its cytopathic effect is unknown, though several mechanisms have been proposed (e.g., accumulation of large amounts of unintegrated viral DNA in infected cells; increase in cell membrane permeability when large amounts of virus bud from the cell surface; speculations that HIV may induce terminal differentiation of infected T4 cells, leading to a shortened life span). There is growing evidence that both the CD4 molecule and the virus envelope play a role in the cytopathic effect in HIV infected cells. A prominent feature in the cytopathology of HIV infection is the formation of multinucleated syncytia which appear to be induced by the gp120/gp41 envelope proteins. In contrast, HIV-infected macrophages may continue to produce HIV without cytopathic effects for long periods of time.

Evidence that monocytes and macrophages play a major role in the pathogenesis of HIV infection is compelling. In addition to engulfing the virus by phagocytosis, some subsets of monocyte-macrophages express the CD4 surface antigen and are therefore capable of binding to the HIV envelope. The monocyte-macrophage is the primary cell type infected in the brain and is involved in the development of the neuropsychiatric manifestations of HIV infection. Moreover, functional defects of monocyte-macrophages are commonly observed in infected patients. These defects may contribute to the opportunistic infections characteristic in

AIDS patients.

Of primary significance is that HIV can survive in a dormant state within the monocyte-macrophage. Infected monocytes do not exhibit the cytolytic effect that HIV has on T4 cells, perhaps due to a lower density of CD4 cell surface receptors. Monocytes can therefore serve as HIV reservoirs which may ultimately transport the virus to the brain, central nervous system, and various organs in the body. It is likely that the virus crosses the blood/brain barrier within monocytes where it affects the release of monokines, enzymes, and chemotactic factors resulting in the destruction or damage of neurons and inflammation of brain tissue (Ho et al., 1987, N. Engl. J. Med. 311: 278; Fauci, 1988, Science 239: 617). Of the various neurologic syndromes directly ascribable to HIV infection, most prevalent is subacute encephalitis or AIDS dimentia (nearly 90% of AIDS patients), the clinical features of which include dimentia, psychomotor retardation, and behavioral changes.

2.3. MORPHOLOGY AND GENOMIC DIVERSITY OF HIV

The fine structure of HIV has been determined by immunological and electron microscopic analytical techniques (Gelderblom et al., 1988, Micron and Microscopia 19: 41; Gelderblom et al., 1987, Virology 156: 171). No morphological difference was detected between HIV-1 and HIV-2 strains (Gelderblom et al., 1988, Micron and Microscopia 19: 41). HIV virion is a spherical particle of about 100 to 120 nm across and contains an electron dense, tubular core comprised of the p24 gag protein, a submembrane matrix comprised of gag p17, and an envelope comprised of the env proteins gp120 and gp41 interspersed within the lipid bilayer membrane. HIV genomic RNA is housed within the core as part of the ribonuclear protein (RNP) complex

which incorporates reverse transcriptase molecules (the enzyme which catalyzes transcription of RNA to proviral DNA) and core proteins. Envelope proteins gp120 and gp41, derived by proteolytic cleavage from the precursor gp160, exist as noncovalently associated complexes embedded in the membrane. These envelope protein complexes are visualized as knobby protrusions having a maximum width of about 14 nm, a height of 9-10 nm, and appear to be arranged in an icosahedral pattern having T=7 laevo symmetry. The protrusions comprise gp120, which is loosely connected to its transmembrane gp41 anchor. Envelope gp120 is spontaneously shed to a high degree from the surface of the virus, a phenomenon which may influence HIV pathogenicity. Virus particle maturation takes place both during and just after the budding process. After budding from the surface of the infected cell, HIV core proteins are cleaved from precursors by an HIV-encoded protease into mature structural proteins which organize to form the core structure.

HIV genome contains three genes that encode the major structural components of the virion: env (which codes for the envelope proteins), gag (which codes for the core proteins), and pol (which codes for reverse transcriptase, protease, and endonuclease enzymes). These three genes are flanked by stretches of nucleotides called long terminal repeats (LTRs). The LTRs include sequences that have a role in controlling the expression of viral genes. However, unlike other retroviruses, the genome of HIV includes at least six additional genes, three of which have known regulatory functions. Expression of these regulatory genes is thought to have an impact on HIV pathogenesis. The tat gene encodes a protein that functions as a potent trans-activator of HIV gene expression and, therefore, plays an important role in the amplification of virus replication. The rev gene product regulates the splicing and transport of

HIV mRNA. In contrast, the nef gene may down regulate virus expression. The vif gene is not absolutely required for virion formation, but is critical to the efficient generation of infectious virions and influences virus transmission in vitro. The Vpr gene encodes an immunogenic protein of unknown function. Lastly, the recently described Vpu open reading frame encodes a protein involved in the regulation of virus maturation and cytopathic effect.

Many different isolates of HIV have been obtained and their nucleotide sequences determined, revealing a striking degree of genomic diversity in the env gene. Regions of the env gene characterized by significant divergence are interspersed with domains conserved among different isolates. Presumably, one such conserved region is the CD4 binding domain, as all HIV isolates bind to the CD4 cell surface receptor molecule. Related but distinct HIV-1 variants, some of which are antigenically diverse, have been isolated from individual AIDS patients over the course of an infection. Isolates may differ with respect to their tropism for specific cell types. In this regard, certain isolates appear to replicate preferentially in either $CD4^+$ T cells or in brain-derived macrophages, suggesting that HIV infection results in different clinical manifestations due to a selective pathogenicity mechanism.

2.4. VACCINE PROSPECTS

Traditional approaches to viral vaccines involve the use of whole virions, either as live attenuated forms or as inactivated preparations. These approaches have been used successfully against many diseases such as smallpox, polio, measles, mumps, rubella, etc. However, the potential usefulness of these approaches applied to HIV vaccine development has been questioned. In addition to the realistic risks associated with reversions and insufficient

activation, such traditional approaches also pose a theoretical risk of inducing a disease such as AIDS since they involve the introduction of the entire retroviral genome into otherwise healthy individuals. Consequently, most efforts toward the development of an AIDS vaccine to date have focused on recombinant approaches, either in the form of subunit or viral vectored vaccines.

Study of HIV target antigens has been largely limited to the envelope glycoproteins and, to a lesser extent, the core antigens. Little, if any, information is known about the immunogenicity of envelope glycoprotein complexes (i.e., gp120-gp41 multimer complex, as opposed to soluble gp120 or gp160) or about envelope-core antigen complexes. Several lines of evidence argue that the presentation of both envelope glycoprotein complex and core antigens in a particular structure may be important. First, studies of the hepatitis B surface antigen indicate that the presentation of that antigen as part of a particle structure is more effective than the soluble antigen (Cabral et al., 1978, J. Gen. Virol. 38:339). Second, the immunogenic property of a given epitope, such as the group-specific neutralizing epitope(s) of adenovirus hexon, may be conformation-dependent. Third, the inclusion of core antigen as an immunogen may elicit broadly reactive immune responses to different HIV-1 isolates since the core antigens of HIV are relatively conserved among various isolates. Therefore, it is of interest and importance to design and evaluate vaccines that combine the advantages of both traditional and recombinant approaches, i.e., recombinant-made vaccines that preserve the immunogenic properties of native virions yet lack the infectivity and other potential disadvantages of whole virus preparations.

In addition to their prophylactic use, vaccines may also be useful for post-exposure immunotherapy. For example,

current rabies vaccines are given to individuals following potential exposure to rabies viruses. It has been proposed that immunotherapy could also be of value in preventing AIDS in HIV infected individuals, since there is a long period of latency between infection and disease progression (Salk, 1987, Nature 327:473-476).

3. SUMMARY OF THE INVENTION

The present invention is directed to nonreplicating recombinant-made retroviral particles, vaccine formulations comprising nonreplicating recombinant-made retroviral particles, methods for the generation of nonreplicating recombinant-made retroviral particles, and the use of nonreplicating recombinant-made retroviral particles as antiviral agents. The recombinant-made retroviral particles of the invention comprise retroviral core and envelope proteins assembled into structures having immunological and morphological characteristics that closely resemble those of native retrovirus virions. The primary structural difference between the recombinant-made retroviral particles of the invention and native retroviral particles is the absence of a complete retroviral genome in the former. Without a retroviral genome capable of directing the expression of, inter alia, the several gene products necessary for retroviral infectivity and replication, the recombinant-made retroviral particles of the invention are totally noninfectious and can not reproduce. Yet, because the recombinant-made retroviral particles are structurally organized as are infectious retroviral particles, they are highly immunogenic and are capable not only of eliciting a protective immune response against the particular retrovirus of interest, but are also effective at blocking retrovirus infectivity.

Applicants' method for generating the nonreplicating

recombinant-made retroviral particles of the invention involves the coexpression of retroviral core and envelope structural proteins in mammalian host cells capable of directing their maturation and supporting their association into correctly assembled budding particles. Introduction of the nucleotide sequences encoding such retroviral core and envelope structural proteins into the mammalian host cell may be accomplished using several established techniques such as, for example, infection by live virus vectors and transfection with DNA vectors. In addition, applicants believe that a nucleotide sequence encoding retroviral protease should also be introduced into the mammalian host cell in order to ensure the proper processing of the retroviral core proteins.

More particularly, the present invention is directed to nonreplicating recombinant-made HIV particles, vaccines against Human Immunodeficiency Virus, methods for generating nonreplicating recombinant-made HIV particles, and the use of nonreplicating recombinant-made HIV particles to inhibit HIV infection and to treat individuals infected with HIV. In a particular embodiment, described by way of example herein, recombinant vaccinia viruses are used as vectors to introduce the gag, protease and envelope genes of Human Immunodeficiency Virus into mammalian host cells which direct the generation of HIV-1-like particles having immunological and morphological characteristics closely resembling those of native HIV-1. These recombinant-made HIV-1 particles are able to block the infectivity of live HIV in vitro and are highly immunogenic in vivo.

4. BRIEF DESCRIPTION OF THE FIGURES

FIG. 1. Radioimmunoprecipitation analysis of HIV-1 proteins expressed in BSC-40 cells infected with recombinant vaccinia viruses. Monolayers of BSC-40 cells were grown to

confluency in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal calf serum (FCS). Cells were infected with either v-env5 (lanes A and B), v-env5 + v-gag2 (lanes C and D), v-gag2 (lanes E and F), or v-NY parental virus (lanes G and H) at a MOI of 10 PFU/cell of each virus. At 12 hours post infection, the cells were radiolabeled for 4 hours with [^{35}S]-methionine and [^{35}S]-cysteine (100 uCi/ml). Culture media was collected and the cells washed with PBS, harvested, and lysed in RIP buffer (1% NP40, 0.5% deoxycholate. 0.1% SDS in PBS). The post-nuclear cell lysates (lanes A, C, E, and G) in parallel with the culture media (lanes B, D, F, and H) were assayed for HIV-1 proteins by RIP with human polyclonal anti-HIV-1 sera (Section 6. 1.2., infra), followed by fractionation by SDS-PAGE in 11.5% acrylamide matrix. Radiolabeled proteins were visualized by autoradiography. Molecular weight markers are indicated in killodaltons (kD).

FIG. 2. Cell surface compartmentalization of the HIV envelope proteins synthesized by infected BSC-40 cells. Cells were infected with v-env5, or coinfected with v-env5 and v-gag2, in parallel with parental v-NY virus at an MOI of 10 PFU/cell for each virus. At 16 hours post infection, the culture media was aspirated and the cells washed and radiolabeled with 0.5 mCi [^{125}I] by the lactoperoxidase catalyzed reaction (Haffar et al., 1987, Mol. Cell. Biol. 7: 1508). Post-nuclear cell lysates were then assayed by RIP and SDS-PAGE for HIV proteins as described in FIG. 1 and in Section 6.1.2., infra.

FIG. 3. Isolation of recombinant HIV particles containing HIV-1 gag and env proteins. (1) Autoradiogram: BSC-40 cells infected as described in FIG. 2 were radiolabeled at 5 hours post-infection with [^{35}S]-methionine and [^{35}S]-cysteine (60 μCi/ml) for 10 hours. The culture media from each infection condition (14 ml) was collected

and clarified of cells by centrifugation at 600 X g for 10 minutes. Two milliliters of the resulting supernatants were collected for assay of the starting material (TS). The remaining 12 ml from each sample was fractionated in a SW 55Ti rotor at 120,000 X g for 3 hours into a particulate pellet and a post-particulate supernatant (S). The pellet was rinsed, resuspended in PBS and overlayed onto a 2 ml 15% sucrose cushion. The particulate material was sedimented again by ultracentrifugation in a SW 55Ti rotor at 120,000 X g for 1.5 hour. The resulting particulate pellet (P) was resuspended in RIP buffer. The P fractions (lanes B, E, and H) were assayed for HIV proteins in parallel with 2 ml (from 12 ml total) S fractions (lanes C, F, and I) and the 2 ml TS material (lanes A, D, and G) by RIP as described in Section 6.1.2., infra. (2) Graph: The particulate pellet from the first ultracentrifugation of doubly-infected BSC-40 culture supernatants was overlayed onto a continuous sucrose density gradient (15%-60%) and sedimented at 120,000 X g in a SW 55Ti rotor for 1.5 hour. The gradient fractions were collected from the bottom of the gradient in 200 ul aliquots. The collected material was halved and assayed for gag p24 content by EIA as described in Section 6.1.2., infra. The peak fractions of the EIA are presented as ng of p24 detected per fraction collected (closed circles), and can be correlated to sucrose concentration (open circles). The fractions constituting the top of the gradient (not shown) did not contain any p24 as confirmed by western blot analysis.

FIG. 4. Analysis of assembled recombinant HIV-1 particles by thin section electron microscopy and immuno electron microscopy. Intact BSC-40 cells coinfected with v-env5 and v-gag2 (MOI=10 PFU/cell for each virus), in parallel with particulate pellet sedimented from culture supernatants (FIG. 3), were fixed for 20 minutes in 4%

paraformaldehyde. The samples were then washed and blocked with 0.8% bovine serum albumin, 0.1% gelatin and 5% normal goat serum in PBS. MAbs 110-4 or 41-1 (Section 6.1.2., infra) as ascites fluid (1:2000 in blocking buffer) were added to the various samples as indicated. After 3.5 hours, the samples were washed with PBS and incubated with gold-conjugated goat anti-mouse IgG and prepared for EM analysis following the procedures outlined in Section 6.1.3., infra.

FIG. 5. Nucleic acid content of recombinant-made HIV-1 particles, determined by dot blot hybridization assay as described in Section 6.2.4., infra. Panel A: gag-specific probe. Panel B: env-specific probe. Recombinant-made HIV-1 particle and inactivated HIV virion concentrations were determined as ng p24 equivalents. (Inactivated virus: lane 1, 2600 ng p24; lane 2, 260 ng p24; lane 3, 26 ng p24; lane 4, 2.6 ng p24; recombinant-made HIV particles: lane 1, 300 ng p24). Panel C: line drawing indicating the coordinates for the gag-pol gene (258-3317) and the env gene (5671-8572) used in the preparation of the recombinant vaccinia viruses v-gag2 and v-env5, respectively. The arrow indicates the position of the RNA packaging sequence (300-319) located upstream of the gag-pol gene (Lever et al., 1989, J. Virol. 63:4085-4087).

FIG. 6. Schematic representation of the construction of plasmid pv-G2E5.

FIG. 7. Western Blot of Recombinant Vaccinia Virus Infected Cells. BSC-40 cells were infected at an MOI of 5 pfu/cell and harvested for PAGE at 24 hours post infection. Infected cell lysates were electrophoresed in a 7-15% acrylimide gel and electro-transferred to nitrocellulose. Immunoblots were reacted with HIV + Human Serum (Trimar) and then peroxidase conjugated goat-anti-human IgG. Blots were visualized using 2-Chloro-Napthol as substrate. Gel lanes were loaded as indicated.

FIG. 8. Radioimmunoprecipitation analysis of recombinant-made HIV-1 particles produced by v-G2E5 infected

BSC-40 cells.

FIG. 9. Schematic diagrams of plasmid vector constructs described in Section 8.1., infra. A: CmHIVdelXmn(1133-a1) and CmHIVdelKpnAvr(Gag2TRE)(1160-a1). B: CmHiGag2Rre(1158-a1) and CmvGag2Rre(1159-a1). C: CmHiEnv5(1104-b1) and CmHiTgfbEnv5(1113-a1). D: BsMkTat(1103-1), BsMtRev(1102-2), CmHiRev(1132-c1) and CmvRev(1152-a1).

FIG. 10. Immunoreactivity of recombinant-made HIV-1 particles generated in transfected CHO cells. Recombinant-made HIV Particles were collected from the culture medium of 3010-C6 cells, concentrated by high speed centrigution and fractionated by banding in a 15-60% sucrose gradient. Top: Gradient fractions were analyzed for Gag protein content by Gag antigen EIA, and for sucrose density using a refracto-meter. Bottom: Aliquots of selected fractions (indicated on top panel by *) were analyzed by electrophoresis on a 7-15% gradient selected fractions polyacrylamide gel and electro-transfer to a nitrocellulose filter which was probed with a human AIDS patient serum and 125-I labeled Protein A. Also loaded were an aliquot of unfractionated particles and isolated HIV virus.

FIG. 11. Analysis of HIV-specific antigens expressed in HeLa cells transfected with plasmid vectors encoding HIV-1 env, tat and rev genes. HeLa cells were contransfected with CmHiTgfbEnv5 (each lane) plus the tat and rev plasmids indicated above each lane ("Bs" is a control plasmid comprised of the "Bluescribe plus" vector with no coding sequences inserted). Zinc was added to cultures, indicated by "+Zn", 24 hours before samples were collected. Total cellular lysates were analysed by electrophoresis on a 10% polyacrylamide gel and electro-transfer to a nitrocellulose filter which was probed with 125-I labeled monoclonal antibody 110-4. Also loaded was an aliquot of BSC-40 cells infected with vaccinia virus v-env5.

FIG. 12. Western Blot of Recombinant Vaccinia Virus Infected Cells. BSC-40 cells were infected at an MOI of 10

pfu/cell and harvested for PAGE at 23 hours post infection. Infected cell lysated were electrophoresed in a 8.5% acrylimide gel and electro-transferred to nitrocellulose. Immunoblots were reacted with HIV+ Human Serum (Trimar) and then peroxidase conjugated goat-anti-human IgG. Blots were visualized using 2-Chloro-Napthol as substrate. Gel lanes were loaded as indicated.

FIG. 13. Analysis of the infectivity of T-lymphoblastoid cells with recombinant-made HIV-1 particles. T lymphoblastoid cells (CEM) were incubated with either recombinant-made HIV-1 particles (corresponding to 3ng p24 gag) or HIV virus (corresponding to 5pg p24 gag), as described in Section 7.1., infra. Five days post infection cell samples were collected from each well and analyzed for intracellular HIV antigens by indirect immunofluoresence, as described in Section 7.1, infra. Evans Blue dye (red stain) was used to facilitate localization of cells. Panel A: CEM cells incubated with recombinant-made HIV-1 particles. Panel B: CEM cells incubated with HIV virus, showing positive fluorescence. Panel C: Syncytium of CEM cells incubated with HIV virus.

FIG. 14. Relative antibody titers in serum samples collected from New Zealand White rabbits immunized with (A) recombinant-made HIV-1 particles, and (B) psoralen-inactivated HIV-1, as determined by ELISA. The details of the assay are set forth in Section 14.1., infra.

FIG. 15. Humoral immune response in immunized animals. New Zealand white rabbits were immunized with recombinant-made HIV-1 particles (R#238 and R#241) and inactivated HIV-1 virions (R#239 and R#243). At different intervals following the primary immunizations serum samples were collected and assayed for HIV-1 specific antibodies by ELISA on disrupted whole virus (panels A and B) or on purified gp120 (panels C and D). The data presented (ordinate) are the end point

titers of HIV-1 specific antibodies calculated at 2 fold the preimmune serum titers. The abscissa values represent the schedule in weeks post-primary immunizations when serum samples were collected. The arrows indicate the times of the secondary (wk4 R#241 and 243, wk5 R#238 and 239) and tertiary (wk18 R#241, wk33 #R238) immunizations.

FIG. 16. Neutralization of HIV-1 infectivity of CEM cells with the rabbit sera. Selected serum samples from the immunized rabbits (panel A R#238 and R#241, panel B R#239 and R#243) were assayed for HIV-1 specific neutralizing activity. The homologous virus (BRU isolate) was preincubated with the appropriate sera for 45 minutes at 37°C prior to addition to the cells. After 1 hour at 37° the virus and sera were removed from the cells and replaced with appropriate dilution of sera in culture medium. Neutralization was determined by measuring, using a EIA, the reduction in $p24^{gag}$ protein released from the cells. The reported neutralizing titers (ordinate) were for 75% reduction in p24 levels in the culture medium. The abscissa values are as described in the description of FIG. 15.

FIG. 17. Antibody reactivity with individual viral proteins as determined by Western blot analysis. Details of the experimental procedure used and discussion of the results are presented in Section 14.2.4., infra.

FIG. 18. Confocal Laser Scanning Micrographs of HeLa cells transfected with CD4 gene incubated with recombinant-made HIV-1 particles then with HIV specific antibodies. Details of the procedure are set forth in Section 15.1., infra.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to nonreplicating recombinant-made retroviral particles which closely resemble live retrovirus virions in their immunological, structural, and morphological features. The method of the invention is

applicable to the construction of recombinant-made particles resembling any of the human retroviruses (e.g., HTLV-I, HTLV-II, HIV-1, HIV-2).

A particular aspect of the invention relates to recombinant-made HIV particles. Recombinant-made HIV particles, like native HIV, are comprised of correctly processed and assembled HIV core and envelope proteins which retain immunoreactivity to anti-HIV sera. The recombinant-made HIV particles do not, however, contain HIV genome and are therefore nonreplicating. The method of the invention is illustrated by examples in which a novel in vitro system is used to generate recombinant-made HIV-1 particles displaying gp120/gp41 envelope protein complexes on their surfaces. One skilled in the art will understand that the present invention encompasses numerous embodiments, and any falling within the scope of the appended claims not specifically described or illustrated herein are within the scope of the invention.

Several features of the present invention distinguish it as a novel approach to an AIDS vaccine. First, the recombinant-made HIV particles closely resemble authentic HIV virions, both in morphology and in antigenic properties. When used as an immunogen, these particles will present antigens in a manner similar to presentation of antigen during HIV infection, thereby eliciting immune responses that are highly relevant and potentially protective against natural infection. This feature of the invention can not be achieved by any recombinant subunit vaccine described to date. Second, the approach of the invention, being based on recombinant DNA techniques, provides flexibility for incorporating antigens from diverse isolates of HIV-1 and HIV-2, thereby generating cross-reactive immune responses considered essential for an effective vaccine against AIDS. Recombinant DNA techniques may also be used to delete or

modify potentially harmful epitopes that contribute to any enhanced infectivity or pathogenicity of the virus. Third, the recombinant-made HIV particles described herein are not infectious and do not contain complete HIV genome. Therefore, immunization with these particles does not introduce the risk of infection potentially associated with inactivated or attenuated whole virus vaccines. These and other features of the present invention are further explained in the sections and subsections that follow.

The recombinant-made HIV particles of the invention may also be useful as specific immunological enhancers to prevent the progression of AIDS in individuals already infected with HIV by boosting the immune reaction against the virus. This embodiment of the invention is directed towards potentiating or maintaining the immunoprotective factors already induced in the seropositive individual. A similar approach is being evaluated by Dr. Jonas Salk using killed, envelope-depleted HIV preparations.

Other contemplated uses of the recombinant-made HIV particles of the invention include their use as an anti-viral agents which interfere with HIV infection, their use in raising monoclonal antibodies to HIV core and envelope protein antigens, their use in the development of anti-idiotypic antibodies, and their use in elucidating the process of HIV encapsidation. The recombinant-made HIV-1 particles of the invention demonstrate antiviral effect (Sections 12 and 13, infra) and elicit HIV-specific humoral and cellular immune responses in both rabbits and macaque monkeys immunized with the particles (Sections 14 and 16, respectively).

5.1. GENERATION OF NONREPLICATING RECOMBINANT-MADE HIV PARTICLES USEFUL AS VACCINES AGAINST THE HUMAN IMMUNODEFICIENCY VIRUS

Applicants' method for generating recombinant-made HIV

particles involves the coexpression of the HIV env-encoded and gag-encoded structural proteins in mammalian cells. The cultured host cells of choice must be capable of synthesizing and correctly processing HIV proteins. Introduction of the env and gag genes into host cells may be accomplished using a variety of established techniques known in the art including infection by live virus vectors, such as vaccinia virus and retroviral vectors, and transfection using DNA vectors. Following successful expression of the HIV proteins in host cells, the recombinant-made HIV particles may be isolated from the culture media using techniques standard in the art.

In a specific embodiment described in further detail below and by way of example in Section 6., infra, recombinant-made HIV particles are produced in African green monkey kidney (BSC-40) cells coinfected with two recombinant vaccinia viruses, one carrying the complete gag gene and the other carrying the complete env gene of HIV-1. This double infection results in the budding of assembled, recombinant-made HIV-1 particles from the surface of the BSC-40 cells. Biochemical analysis revealed that these particles incorporated mature, immunoreactive gag and env proteins. The morphology of the recombinant-made particles, as visualized by electron microscopy, is virtually the same as live HIV.

In a related embodiment, an alternative system for generating the recombinant retroviral particles of the invention using viral vectors is demonstrated by way of examples in which a single recombinant vaccinia virus containing both the env and gag genes of HIV-1 is used to transfect mammalian cells which then generate recombinant-made HIV-1 particles (Section 7, et seq., infra).

In another embodiment, recombinant-made retroviral particles may be generated using a system involving

mammalian cells transfected with DNA encoding the retroviral structural proteins. As one example of this embodiment, described in further detail in Section 8, et seq., infra, two plasmid vectors encoding, respectively, the HIV-1 gag and HIV-1 env genes, are used to transfect CHO cells, which then direct the synthesis of HIV-1 gag and env antigens assembled into recombinant-made HIV-1 particles. Other strategies in connection with this embodiment include, but are not limited to, transfection with complex plasmid vectors containing multiple HIV genes, the use of constitutive and regulatable enhancer/promoter elements to drive the expression of HIV genes, the expression of HIV regulatory proteins in conjunction with and to control, HIV structural gene expression, the use of different cell lines, and the use of plasmid vectors encoding modified HIV proteins.

Other strategies for the expression of HIV structural proteins and the generation of recombinant-made HIV particles are disclosed in, for example, Sections 8.3., 9., et seq., and 10, et seq., infra.

A number of options are available using the systems of the present invention that allow control over the nature of the resulting particles. These options may be exercised at both the virus construction and infection stages.

5.1.1. PREPARATION OF RECOMBINANT DNA AND VIRAL VECTORS

Recombinant DNA vectors and viral vectors such as vaccinia viruses may be constructed according to the methods outlined in copending United States Patent Applications Serial No. 779,909 filed September 25, 1985; Serial No. 842,984 filed March 27, 1986; and Serial No. 905,217 filed September 9, 1986, each of which is incorporated by reference herein in its entirety.

In a particular embodiment of the invention, recombinant

vaccinia viruses carrying HIV env and gag sequences are constructed and used as vectors. Briefly, plasmid vectors containing HIV core and envelope protein coding sequences under the transcriptional control of the vaccinia promoter are constructed and used to affect the integration of the HIV gene sequences into the vaccinia virus genome by in vivo recombination. Recombinant vaccinia viruses are identified, purified, and evaluated for their ability to direct the synthesis of HIV proteins in infected cells; as described in the above-referenced copending patent applications.

In another embodiment, recombinant plasmid vectors encoding various combinations of HIV structural and/or regulatory genes are constructed and used as vectors for transfecting cells capable of generating recombinant-made HIV particles. The constructions of a representative range of such vectors are described in Section 8., et seq., infra.

The precise nature of the individual protein components of the recombinant-made particles of the invention may be modified by recombinant DNA techniques, during construction of the recombinant DNA vectors, recombinant vaccinia virus vectors, etc. In this way, the existence and structural composition of retroviral epitopes presented on the particles may be defined. For example, variable epitopes of HIV gp120 from the different HIV isolates may be included to generate a cross-reactive immune response. Similarly, different HIV gag gene sequences may be incorporated within recombinant vectors to vary the immunogenicity of recombinant-made HIV particles. Vectors encoding mutated HIV gene sequences may also be useful in generating recombinant-made HIV particles, which may result improved in immunogenicity, anti-viral effect, etc. Applicants intend that recombinant-made retroviral particles incorporating such modified core and/or envelope proteins be within the scope of the present invention and the appended claims.

5.1.2. INFECTION/TRANSFECTION OF HOST CELLS WITH RECOMBINANT VECTORS TO GENERATE RECOMBINANT-MADE RETROVIRAL PARTICLES

The nature of the recombinant-made retroviral particles is controlled to a large extent not only by the composition of the recombinant vectors used but also by the combination of vectors used in the infection or transfection process, this choice being a primary variable in the overall method of the invention. As a simple illustration, applicants have discovered that infecting BSC-40 host cells with a single recombinant vaccinia strain carrying the HIV-1 gag gene (v-gag2) resulted in the formation of HIV-1 core proteins assembled into particles. However, when these same cells were coinfected with v-gag2 as well as a recombinant vaccinia carrying the HIV-1 env gene (v-env5), the resulting assembled particles incorporated and displayed HIV-1 envelope proteins on their surfaces (Section 6., infra). Thus, while a particle comprised only of core proteins is formed after infection with v-gag2, adding v-env5 to the infection system results in particles of greater structural complexity which incorporate the envelope proteins. Taking the above illustration one step further, coinfection of host cells with v-gag2, v-env5, and a third recombinant vaccinia carrying the env gene from HIV-2 would be expected to result in the formation of heterologous particles incorporating HIV-1 core as well as both HIV-1 and HIV-2 envelope proteins. Alternatively, single recombinant vaccinia viruses encoding multiple HIV genes may be used as infection vectors. As demonstrated by example in Section 7, et seq., infra, host cells infected with one such vaccinia virus vector also generate immunoreactive recombinant-made HIV-1 particles.

The same principles apply to generating recombinant-made particles using other vector systems, such as the use of plasmid vectors to transfect host cells. For example, host

cells may be transfected with a single plasmid vector encoding both HIV env and gag, or a vector encoding HIV env, gag and other HIV genes, etc. Cells may also be transfected with a plurality of plasmid vectors, each encoding a different HIV gene or combinations of HIV genes. Furthermore, transfected cell lines may be transfected again with vectors designed to add the expression of other HIV genes to the particle generation system. Vectors encoding regulatable promoters may be used to modulate the expression of HIV proteins in transfected host cells (see, for example, Section 8.3.2., infra). Vectors encoding other HIV genes may be used to affect their expression in conjunction with the expression of HIV structural genes in transfected cells. The expression of such HIV regulatory and/or accessory proteins in the system may be used as a means of altering particle characteristics and/or their production levels.

It is important to note that particles will not form in the absence of gag proteins, thus the inclusion of essential HIV core protein gene sequences in the recombinant vector is necessary. Although HIV protease is not required for the assembly of core particles, its role in the formation of infectious virion is indicated (Peng et al., 1989, Virology 63: 2550). As such, it may be advantageous to include HIV protease function in the production of recombinant-made HIV particles such that they may closely resemble native virions. In addition, gag genes from HIV-1, HIV-2, or different isolates thereof may be used to construct recombinant vectors. As will be clear to one skilled in the art, the multi-infection approach illustrated above, and similar approaches using other recombinant vectors, may be used to generate recombinant HIV particles having a broad range of surface and core antigen characteristics. The number of different combinations are essentially unlimited.

The particular host cell selected will also influence

the nature of the particles produced by the method of the invention. Cells should be chosen for their ability to express and correctly process mature HIV proteins. Since the HIV envelope proteins gp120 and gp41 are glycosylated and are derived by proteolytic cleavage from a larger gp160 precursor, a cell capable of directing these post-translational processing modifications is desirable. Of course, where recombinant viral vectors are employed, the host cell must be susceptible to infection with recombinant virus. In preferred embodiments of the invention, host cells of human, simian, or rodent origin are used.

Host cells may be infected by recombinant vaccinia virus according to the conditions described in Section 6., infra, or transfected by recombinant plasmid vectors as described in Section 8., et seq., infra. When utilizing a recombinant vaccinia vector system, such as that described in Section 6., et seq., infra, cells may be infected at a multiplicity of infection (MOI) of about 10 PFU per cell of each recombinant vaccinia virus. However, one skilled in the art will understand that increasing or decreasing the MOI for one or more recombinant vaccinia may be used to influence the nature of the resulting particles. This would be an important factor in designing polytropic or heterologous particles. For example, a desired ratio of HIV-1 to HIV-2 envelope antigens on the surface of a heterologous particle may be achieved by infecting with a proportionally corresponding MOI ratio.

In a specific embodiment of the invention, described in detail in Section 6., infra, recombinant vaccinia viruses v-env5 and v-gag2 were used to coinfect cultured African green monkey kidney (BSC-40) cells. The infected BSC-40 cells synthesized HIV-1 envelope proteins gp120, gp41, and the gp160 precursor, as well as HIV-1 gag proteins p24, p17, p15, p55, p45, and p39. Furthermore, at least p24, p17,

gp120 and gp41 assemble particles that are immunoreactive to polyclonal anti-HIV-1 sera and monoclonal antibodies specific for p17, p24, gp120 and gp41. Ultrastructural analysis of the recombinant-made HIV-1 particles by thin section electron microscopy and immunogold labeling revealed substantial morphologically identity with native HIV. In this regard, recombinant-made HIV-1 particles were visualized as spherical objects having a diameter of between 100 and 120 nm, and contained an electron dense inner core which was either rod-shaped or spherical, depending on the section angle. Capture enzyme immunoassay and immuno electron microscopic analysis using monoclonal antibodies against p24 and p17, respectively, confirm the identity of the core structures of HIV. Immuno electron microscopy analysis using monoclonal antibodies specific for HIV-1 gp120 and gp41 demonstrated that the gp120/gp41 complex is displayed on the surface of the recombinant-made HIV-1 particles. Various forms of immature particles were also visualized, consistent with the course of HIV-1 virion morphogenesis. These features virtually parallel those observed from similar analyses of the HIV-1 virion (Compare the electron micrographs shown in FIG. 4 with those presented in Gelderblom et al., 1988, Micron and Microscopia 19: 41, and in Gelderblom et al., 1987, J. Virol. 156: 171). Applicants' results suggest that the ultrastructure of these recombinant-made HIV-1 particles differs from the ultratructure of live HIV-1 only in that they do not contain the viral genome or the reverse transcriptase enzyme.

The recombinant-made HIV particles of the invention may also be generated by cells transfected with recombinant plasmid vectors encoding HIV gag, env, and other HIV genes. Various specific embodiments of this aspect of the invention are described in Section 8.2. infra. In a particular embodiment, Chinese Hamster Ovary (CHO) cells are

assays, including whole virion ELISA, gp120 ELISA, focal immunoassay and lymphoproliferative response assay. As described by way of the examples in Section 16., et seq., infra, when used as the sole immunogen for primary and secondary immunizations, recombinant-made HIV-1 particles elicit HIV-specific humoral and cellular immune responses. The recombinant-made HIV-1 particles were particularly effective when used to immunize animals previously primed with a recombinant vaccinia virus encoding HIV-1 env and gag antigens.

5.4. VACCINE FORMULATIONS

A specific embodiment of the invention is the formulation of vaccines capable of invoking immune responses that contribute to the prevention of retrovirus infection or the development of retrovirus-associated diseases such as AIDS. The vaccine formulations use the recombinant-made retroviral particles of the invention as immunogens which, by combining major retroviral core and envelope proteins, are multivalent in nature. In related embodiments, the recombinant-made retroviral particles may be used as specific immunologic enhancers that may be used to ameliorate the progression of retrovirus-associated diseases in persons already infected with retrovirus.

5.4.1. VACCINES AGAINST THE HUMAN IMMUNODEFICIENCY VIRUS

A further particular embodiment of the invention involves vaccine formulations capable of invoking immune responses that contribute to the prevention of HIV infection or the development of AIDS. Such vaccine formulations utilize recombinant-made HIV particles as immunogens.

Traditionally, viral vaccines have been prepared from attenuated or inactivated whole virions. Neither approach has been favored for the design of a vaccine against HIV-1

primarily because of the hazards associated with large scale preparation of the virus, potentially incomplete activation, and the introduction of the HIV genome into healthy recipients (Minor, 1989, J. Antimicrobial Chemotherapy 23, Supp. A: 55). HIV vaccine development has therefore focused on subunit vaccine candidates. Although initial attention was directed to a subunit formulation comprising recombinant gp120 envelope protein, it is now clear that both gp120 and gp 41 are target antigens for the development of neutralizing antibodies (Chahn et al., 1986, EMBO J. 5: 3065; Ho et al, 1987, J. Virol. 61: 2024; Skinner et al., 1988, J. virol. 62: 4195), for mediating antibody cellular cytotoxicity (Tyler et al., 1989, Fifth International Conference on AIDS, Abstract T.C.O. 33: 521), and for conferring susceptibility of cytotoxic T lymphocyte killing (Zarling et al., 1987, J. Immunol. 139: 988). These results argued for the inclusion of both gp120 and gp41 in the design of an HIV-1 vaccine.

The association of gp41 with cell membranes, in conjunction with its weak noncovalent interaction with gp120, render impractical the purification of intact soluble gp120/gp41 complexes. More importantly, like other viral antigens presented as components of membrane structures, such as Hepatitis B surface antigen (Cabral et al., 1978, J. Gen. Virol. 38: 339) and Herpes Simplex Virus glycoprotein (Ho et al., 1989, J. Virol. 63: 2951), membrane bound gp120/gp41 complexes are likely to be more immunogenic than the soluble counterparts.

As discussed in the Background of the Invention section herein, development of an effective HIV vaccine is complicated by several characteristics of HIV. The present invention may circumvent many, if not most of the problems. In addition to presenting gag and env proteins in their native conformations, the recombinant-made HIV particles of

the invention may be designed so that desirable epitopes are retained, while undesirable epitopes are deleted or modified. Furthermore, the invention provides a means by which several different variable epitopes of the same HIV protein may be incorporated into the particles used in the vaccine formulation. The invention also provides a way to create heterologous recombinant-made HIV particles that may be used to formulate a vaccine capable of preventing infection by both HIV-1 and HIV-2.

One of the novel aspects of a vaccination approach utilizing the present invention is that a full battery of these and other such epitopes may be consolidated into one immunogenic particle. Moreover, these epitopes are presented to the immune system as they are on native HIV, thereby inducing immune responses that are effective against infection by native virions.

5.4.1.1. HIV-1 VACCINES

Protective immunity against HIV has not been fully elucidated. It is commonly believed that both neutralizing antibodies and cell-mediated immunity may be required, since HIV can be transmitted in cell-free or cell-associated form. Neutralizing antibodies recognizing a number of different HIV epitopes have been identified in HIV-1 infected individuals, including those which recognize epitopes on highly conserved as well as variable regions of the envelope protein gp120. Similarly, neutralizing antibodies directed against epitopes of gp41 and p17 (Papsidero et al., 1989, J. Virol. 63:267-272) have been identified. Other antibodies detected in HIV infected individuals include those specific for domains of gp120 which bind to the CD4 cell surface receptor. Still other antibodies, which bind to a hypervariable region of gp120, are capable of inhibiting fusion of HIV infected cells into syncytia (Rusche et al.,

1988, Proc. Natl. Acad. Sci. U.S.A. 85: 3198). Cellular immune responses are also seen in HIV infected persons and, specifically, cytotoxic T-lymphocytes directed against env, gag and pol gene products have been identified (Walker et al., 1987, Nature 328:345; Nixon et al., 1988, Nature 336:484; Riviere et al., 1989, J. Virol. 63:2270-2277).

One embodiment of the invention is a vaccine against the HIV-1 virus, presently the most prevalent form of HIV, using recombinant-made HIV-1 particles such as the recombinant-made HIV-1 particles described in Section 6., et seq., infra, which comprise mature core and envelope proteins of the type 1 virus, assembled within a structure that mimics the morphologic and antigenic properties of live HIV. This embodiment encompasses vaccine formulations using a variety of recombinant-made HIV-1 particles. For example, particles silmultaneously presenting the gp120/gp41 complexes of two or more HIV isolates may be useful for inducing the development of protective antibodies against a number of variable region epitopes. The generation of such particles could be achieved by coinfecting host cells with several different recombinant vaccinia viruses carrying, respectively, the env genes from the different HIV-1 isolates. Individuals immunized with such particles would be able to mount immune responses against a number of different HIV-1 strains.

Similarly, the construction of polytropic particles may also increase the efficacy of the vaccine formulation. In this embodiment, particles are designed to incorporate epitopes from HIV-1 strains having different tropisms. For example, recombinant-made HIV-1 particles displaying determinants unique to a monocyte-associated HIV-1 strain in combination with determinants common to T4 cell-associated virus strains may be generated using a multi-infection approach. Specifically, such particles may be generated by

coinfecting host cells with three recombinant vaccinia, one carrying the gag gene from one strain, one carrying the env gene from the same strain, and the other carrying the env gene from a tropogenically different strain.

The most effective vaccine against HIV-1 may involve using the recombinant-made HIV-1 particles of the invention in combination with other immunogens. In this regard, recombinant-made HIV-1 particles appear most effective at eliciting humoral and cellular immune responses when used as a secondary immmunogen following initial immunization with recombinant gp160 in non-human primate subjects.

5.4.1.2. HIV-2 VACCINES AND HETEROLOGOUS VACCINES

Other embodiments of the present invention relate to vaccines against HIV-2 as well as heterologous vaccines which may include, for example, a single vaccine for both HIV-1 and HIV-2. The recombinant-made particles used in such heterologous vaccines may comprise, for example, the core proteins of HIV-1 and the envelope proteins of HIV-1 and HIV-2. Alternatively, it may be desirable to formulate a vaccine comprised of two or more different recombinant-made HIV particles. This may be especially true when designing a vaccine that would protect against the many different isolates of both HIV-1 and HIV-2.

Vaccines comprising a single recombinant-made HIV particle type or different types in combination may be formulated with a suitable adjuvant in order to enhance the immunological response to their antigens. Suitable adjuvants include, but are not limited to, mineral gels, surface active substances such as lysolecithin, plurionic polyols, polyanions, peptides, oil emulsions, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and corynebacterium parvum.

A number of methods well known in the art may be used to

introduce the vaccine formulations described above, including intradermal scarification, intravenous injection, subcutaneous injection, intramuscular injection, intranasal administration, oral administration, etc.

6. EXAMPLE: GENERATION AND ISOLATION OF NON-NONREPLICATING RECOMBINANT-MADE HIV-1 PARTICLES

Described here is an in vitro system for generating recombinant-made HIV-1 particles which contain assembled core and envelope proteins and which display the env gp120 and gp41 antigens on their surfaces. Briefly, BSC-40 cells are coinfected with recombinant vaccinia viruses carrying either the complete envelope gene of HIV-1 (v-env5) or the complete HIV-1 gag and protease genes (v-gag2). HIV-1 proteins are expressed in the BSC-40 cells and assemble into HIV-1 particles which bud from the cell membrane. The resulting particles are nonreplicating, react with monoclonal antibodies specific for HIV-1 envelope and core proteins, and are morphologically similar to HIV-1 virions.

6.1. GENERAL PROCEDURES

6.1.1. CELLS AND VIRUSES

African green monkey kidney cells (strain BSC-40, a continuous line of African Green Monkey Cells derived from BSC-1 cells, ATCC No. CCL26) were propagated in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum and 100 units per ml each of penicillin and streptomycin.

Recombinant vaccinia viruses carrying HIV-1 env and gag gene sequences were prepared and evaluated as described in copending United States Patent Application Serial No. 905,217 filed September 9, 1986. Recombinant vaccinia virus v-env5 carries the entire env gene of HIV-1. Recombinant vaccinia virus v-gag2 carries the entire HIV-1 gag and prt

resedimented by ultracentrifugation at 120,000 xg through a 15% sucrose solution layer. The twice sedimented material was refered to as the particulate fraction (P).

The sedimented particulate pellet (P) was washed several times by gently overlaying the pellet with PBS and then aspirating it off. The pellet was then fixed with 4% paraformaldehyde for 20 minutes and washed again with PBS. In a similar procedure, small monolayers of BSC 40 cells (10^4-10^5 cells) were infected with the appropriate recombinant viruses. After 15 hr the growth media was discarded, and the cell monolayers were washed several times with PBS and then fixed with 4% paraformaldehyde for 20 minutes at 22°C.

The fixed cells and fixed particulate pellets were then washed 5 times with PBS and then blocked with a solution containing 0.8% bovine serum albumin (BSA), 0.1% gelatin and 5% normal goat serum in PBS (blocking buffer) for 30 minutes. Blocking solution was decanted and monoclonal antibodies added to the cells as ascites fluid (diluted 1:2000 in blocking buffer) and allowed to incubate for 2 to 3 hours. Cells were washed with PBS and then incubated with gold-conjugated goat anti-mouse secondary antibody (IgG conjugated to 15 nm colloidal gold; Janssen, Piscataway, NJ) at a dilution of 1:5 for an additional 2 hours, washed with PBS, and fixed in 2% glutaraldehyde. Samples were incubated with 1% osmium tetraoxide (OsO_4) in 0.1 M Cacadylate buffer for 30 minutes at 22°C. Samples were then rinsed well with PBS, and dehydrated by 3 minutes sequential incubations in 35%, 50%, and 75% ethanol, prior to staining with 3% uranyl acetate in 70% ethanol for 30 minutes at 22°C. Samples were then further dehydrated with sequential incubations, as described above, in 80%, 90%, 95%, and finally 100% ethanol (3 times), at 22°C.

Samples were then embedded in methacrylate resin

(plastic) as follows: 1 hour treatment with absolute ethanol/plastic at a ratio of 2:1 respectively, overnight treatment with 100% plastic. The resin was then polymerized for 2 days at 60 degrees C. The plastic with the embedded samples was mechanically removed from the dish and thin sections (100 nm) were cut and collected on formvar coated grids. The grids were subsequently stained with saturated uranyl acetate/lead citrate (Millonig's) for 10 minutes each and washed extensively. The grids were evaluated after drying at 100,000 X magnification with a JEOL 100B transmission electron microscope at 60 kV.

6.2. GENERATION OF NONREPLICATING RECOMBINANT-MADE HIV-1 PARTICLES CONTAINING MATURE gag and env PROTEINS

6.2.1. ANALYSIS OF HIV-1 PROTEINS EXPRESSED IN RECOMBINANT VACCINIA VIRUS INFECTED BSC-40 CELLS

Cell lysates and growth media from metabolically radiolabeled BSC-40 cells infected with either v-env5, v-gag2, both recombinants together, or parental vaccinia virus v-NY were analyzed for HIV-1 proteins by RIP with human polyclonal anti-HIV-1 sera as described in Section 6.2.1., supra. As shown in FIG. 1, the gp160 env precursor protein, as well as its proteolytic processing products gp120 and gp41, were detected in the cell lysates of v-env5 infected cells (lane A). Additionally, gp120 was secreted by the v-env5 infected cells (lane B). The p55 gag precursor synthesized in v-gag2 infected cells is also processed to yield the mature gag proteins p24, p17, p15, as well as two intermediate precursor species p45 and p39 (lane E) (Gowda et al., 1989, citation). Interestingly, the gag proteins were also detected in the culture supernatant (lane F).

BSC-40 cells coinfected with v-env5 and v-gag2 yielded processed env and gag proteins identical to those expressed

in cells infected individually (lane C, compare with lanes A and E). The mature env and gag proteins generated by these doubly infected cells were similarly localized to the culture supernatants (lane D, compare with lanes B and F) with identical kinetics. Additionally, lactoperoxidase catalyzed iodination of plasma membrane-associated proteins revealed that the transport of gp160, gp120 and gp41 to the cell surface is the same in v-env5 infected cells as it is in doubly infected cells (Fig. 2, lanes A and B, respectively).

6.2.2. ISOLATION OF NONREPLICATING RECOMBINANT-MADE HIV-1 PARTICLES

To determine whether extracellular gp120 and gp41 expressed by infected BSC40 cells are released as soluble proteins or as constituents of budding particles, culture supernatants were separated by into particulate (P) and post-particulate (S) fractions (Section 6.1.3., supra) and the HIV protein content of each fraction analyzed by RIP in parallel with unfractionated culture supernatants (TS). As shown in FIG. 3(1) (lanes A, B and C), the extracellular gp120 derived from v-env5 infected cells was detected primarily in the S fraction, suggesting that the protein is not a constituent of budding particles. However, gp120 derived from cells coinfected with v-env5 and v-gag2 was detected in the P fraction as well as the S fraction (FIG. 3(1), lanes E and F respectively), though the particulate-associated gp120 contributed little to the overall level of detectable extracellular gp120.

In contrast to gp120, gp41 was detected only in the P fraction of supernatants from doubly infected cells (FIG.3(1), lanes E vs D and F), indicating that gp41 is associated only with budding particles. Of interest is that the gp160 env precursor was detected in the P fraction of supernatants from doubly infected cells, albeit at low

levels (FIG. 3(1), lanes E vs D and F).

HIV-1 core proteins expressed by infected BSC40 cells were analyzed similarly. All detectable core proteins--p24, p55, p45, p39, and p17 -- were observed in the P fractions from v-gag2 infected (FIG. 3(1), compare lanes H and I) as well as doubly infected (FIG. 3(1), compare lanes E and F) cell culture supernatants. P fractions obtained from doubly infected cells were also subfractionated by sedimentation through a continuous sucrose density gradient of 15%-60% and the presence of p24 antigen evaluated by EIA as described in Section 6.1.2., supra. The results depicted in FIG. 3(2) show that p24 partitions as a single prominent peak together with the 36%-40% sucrose fractions. These results suggest that the gag proteins expressed by infected BSC40 cells are released as constituents of particles of relatively uniform size. Moreover, the presence of gp120 and gp41 in the P fractions of doubly infected cells suggests that the particles released from coinfected cells incorporate gp120/gp41 complexes. This conclusion is further supported by the results obtained from immuno electron microscopy analysis (Section 6.2.3., infra).

6.2.3. ULTRASTRUCTURAL ANALYSIS OF RECOMBINANT-MADE HIV-1 PARTICLES BY THIN SECTION ELECTRON MICROSCOPY

To directly analyze the morphology of the recombinant-made HIV-1 particles, P fractions isolated from the culture supernatants of BSC40 cells coinfected with v-env5 and v-gag2 were reacted with monoclonal antibodies (MAbs) specific for either gp120 (MAb 110-4) or gp41 (MAb 41-1). Primary antigen-antibody complexes were further reacted with a secondary antibody-colloidal gold conjugate, embedded in plastic, and viewed by thin section electron microscopy (EM) as described in Section 6.1.3., supra. These analyses revealed particles of about 100-120 nm in diameter containing an electron dense rod-shaped (FIG. 4, "a" and

"c") or spherical (FIG. 4, "b") core morphologically similar to EM images of isolated HIV-1 virion particles (Gelderblom et al., 1988, Micron and Microscopia 19:41; Gelderblom et al., 1987, Virology 156: 171). Moreover, the particles were labeled with the colloidal gold conjugates indicating that they display gp120 (FIG. 4, "a" and "b") and gp41 (FIG. 4, "c") on their surfaces.

Intact BSC-40 cells coinfected with the two recombinant vaccinia viruses were similarly analyzed using the anti-gp120 MAb. Numerous 100-120 nm particles positive for the gp120 antigen were visualized. The majority of these cell associated particles assumed either of two distinct morphologies, one characterized by a diffuse vesicle form (FIG. 4, "e", open arrow) and the other distinguished by an eccentrically-localized, thickened double-membrane region (FIG. 4, "d", double arrow). Applicants speculate that these different structures represent various forms of immature particles which may parallel those occurring during HIV-1 virion morphogenesis. Occasionally, particles containing a fully-formed rod-shaped capsid structure could be seen near the cell surface, as captured in the electron micrograph presented in FIG. 4, "e" (open arrow). Vaccinia particles budding from the cell membrane did not incorporate gp120 (FIG. 4, "d", double arrow).

6.2.4. NUCLEIC ACID CONTENT OF RECOMBINANT-MADE HIV-1 PARTICLES

The nucleic acid content of the recombinant-made HIV-1 particles was determined by dot blot hybridization with ^{32}P RNA probes reactive with either the gag or env sequences. The probes were synthesized by *in vitro* transcription of DNA templates, carrying the gag or env sequences, in the presence of radiolabeled nucleotides using the Promega Riboprobe *in vitro* transcription kit according to manufacturers directions. Briefly, recombinant particles,

equivalent to 300ng p24, were solubilized in RNA preparation lysis buffer (2M guandin isothiocyanate, 125mM sodium citrate pH 7.0, 0.125% sarkocinate, 50% dimethyl sulfoxide) and blotted onto nitrocellulose filters, in parallel with various concentrations of similarly solubilized Psoralin-inactivated HIV (equivalent to 2600ng, 260ng, 26ng, and 2.6ng p24). Separate filters were prepared for reaction with the gag specific or env specific probes. The nitrocellulose filters were incubated for 2 hour at 42°C in hybridization buffer (3x SSC, 50% Formamide, 5x Denhardt's solution, and 150ug nonspecific RNA), prior to addition of the respective probes. The filters were incubated with the probes overnight at 42°C, then washed extensively with 0.1x SSC/0.1% SDS solution, air dryed, and analyzed by autoradiography.

Autoradiograms of the dot bot hybridizations are shown in FIG. 5. Only the gag probe reacted with nucleic acids in the recombinant particle sample (panel A vs panel B). In contrast, both probes reacted with the Psoralin inactivated virus controls. This data suggests that the recombinant-made HIV-1 particles packaged gag but not env RNA. This conclusion is consistent with the fact that the gag gene used for generating the v-gag2 vaccinia recombinant virus included the 5' untranslated sequences recently identified as the packaging signal for HIV viral RNA (FIG. 5, panel C, arrow).

These results indicate that the recombinant-made HIV-1 particles do not have the capacity to replicate. This conclusion is supported by the experiments described in Section 12., infra, which demonstrate the absence of intracellular HIV antigens in $CD4^+$ cells incubated with recombinant-made HIV-1 particles for an extended period.

7. EXAMPLE: GENERATION OF NONREPLICATING RECOMBINANT-MADE HIV-1 PARTICLES USING A SINGLE RECOMBINANT VACCINIA VIRUS VECTOR

As an alternative to the system for generating recombinant-made HIV-1 particles using two recombinant vaccinia virus vectors as described in Section 6., supra, a single viral vector approach involving the construction of a recombinant vaccinia virus containing both the env and gag genes of HIV-1 may be used. In the following example, a recombinant vaccinia virus containing HIV-1 env and gag genes is used to infect BSC-40 cells. The infected cells express envelope and core antigens of HIV-1, which assemble in the infected cells to generate the recombinant HIV-1 particles. Recombinant HIV-1 particles purified from the infected cell media are immunogenic in vivo.

7.1. GENERAL PROCEDURES

7.2. CONSTRUCTION OF v-G2E5: A RECOMBINANT VACCINIA VIRUS CONTAINING HIV-1 ENV AND GAG GENES

A 3.2 Kbp fragment which contains the entire env-coding sequence of HIV-1 (nucleotide no. 5707-8608) under the control of vaccinia virus 7.5K promoter was excised from plasmid pv-env5 (copending United States Patent Application Serial No. 07/593,401, filed October 5, 1990) by restriction endonuclease EcoRI. This fragment was inserted into the EcoRI site on plasmid pv-gag2N (identical to pv-gag2 described in copending United States Patent Application 07/593,401, filed October 5, 1990, with the exception that a synthetic linker containing translational termination signals was inserted at the SmaI site downstream of the gag-coding sequence). Plasmid pv-gag2N contains the HIV-1 gag gene also under the control of vaccinia virus 7.5K promoter. The construction of the resulting plasmid, pv-G2E5, is schematically represented in FIG. 6. The HIV-1 gag and env genes were introduced into the thymidine kinase gene of vaccinia virus by in vivo recombination as described in

copending United States Patent Application Serial No. 07/593,401, filed October 5, 1990, thereby generating recombinant vaccinia virus v-G2E5, containing both gag and env genes of HIV-1.

7.3. COEXPRESSION OF HIV-1 ENVELOPE AND CORE ANTIGENS IN BSC-40 CELLS INFECTED WITH v-G2E5

African green monkey kidney cells (BSC-40) were infected with recombinant v-G2E5 at a multiplicity of infection of 5 pfu/cell. At 24 hr after infection, cells were washed and cell lysates were resolved by SDS-PAGE on a 7-15% gel. Proteins in cell lysates were transfered onto nitrocellulose filters and reacted with HIV-positive human sera, followed by goat anti-human immunogloblin antibodies conjugated to horseradish peroxidase. Immunoreactive proteins were detected by reaction with peroxidase substrates. The results shown in FIG. 7 indicate that both envelope and core antigens of HIV-1 were expressed and processed in cells infected with v-G2E5. The level of both antigens was comparable to that obtained in cells infected with either the env-containing or gag-containing recombinant vaccinia viruses alone.

7.4. RECOMBINANT HIV-1 PARTICLES GENERATED IN v-G2E5 INFECTED BSC-40 CELLS

Recombinant v-G2E5 was used to produce HIV-1-like particles from BSC-40 cells. BSC-40 cells were seeded onto Cytodex 3 beads (Pharmacia LKB Biotechnology) and were grown in spinner culture bottles in Dulbecco modified Eagle's medium with 5% fetal calf serum according to the manufacturer's recommendations. When cells attained confluency, they were infected with recombinant vaccinia virus v-G2E5 at a MOI of 5 pfu/cell. Following a 1 hr adsorption, the cells were washed twice with fresh medium to remove any excess inoculum. After 24 hr incubation at 37.5°C, culture medium was collected and cell debris removed

by low speed centrifugation. Pre-cleared culture medium was then centrifuged for 3 hr at 19,000 rpm in a type 19 rotor (Sorvall). Pellets from this high-speed centrifugation were resuspended in PBS, pooled and resedimented by centrifugation for 1 hr at 32,000 rpm using a SW55Ti rotor (Beckman). The final pellet was resuspended in cold PBS and stored at -70.5°C until use. The p24 and gp120 antigen contents of the particle preparations were quantitated by p24 antigen capture and immunoblot assays, respectively, essentially as described in Section 6.1.2., supra.

A radioimmunoprecipitation analysis of the particles produced by v-G2E5 infected cells is represented in FIG. 8. These results demonstrate the presence of both gp120 and p24 antigens in the pellet fraction of infected cell medium after high-speed centrifugation, indicating the selective assembly of these mature virion proteins into particle forms. The relative abundance of p24 and gp120 in these particles were similar to that of HIV-1 virion preparations, underlying the structural and biochemical similarities between recombinant-made and authentic HIV-1 virions.

8. EXAMPLE: GENERATION OF NONREPLICATING RECOMBINANT-MADE HIV-1 PARTICLES BY TRANSFECTION OF PLASMID DNA INTO MAMMALIAN CELLS

In addition to the systems for generating recombinant HIV-1 particles using recombinant vaccinia virus vectors, described in Sections 6 and 7, supra, a system involving mammalian cells transfected with DNA encoding HIV-1 env and gag genes may be used. This system allows for the generation of recombinant HIV-1 particles in a stable mammalian cell line.

Generation of recombinant-made HIV particles using the system described herein may be accomplished using a variety of strategies, including but not limited to (1) the transfection of complex plasmid vectors containing multiple HIV structural or regulatory genes, (2) the co-transfection

of multiple plasmid vectors containing different HIV genes, (3) the use of both constituitive and regulatable enhancer/promoter elements to drive the expression of HIV proteins, (4) the use of HIV regulatory proteins including tat and/or rev to indirectly control the expression of HIV gag and env structural proteins, (5) the use of different cell lines for expression of HIV proteins and recombinant-made HIV particles, and (6) the use of plasmid vectors encoding modified HIV proteins. These variations allow for the optimization of the system in different cell types and the manipulation of different protein species or structures in the recombinant HIV particles.

8.1. PLASMID CONSTRUCTS

Many of the plasmid vectors used in this system contain a hybrid CMV:HIV Enhancer:Promotor (designated CmHi) derived from the expression vector pH3MPy (Aruffo and Seed, 1987 Proc. Natl. Acad. Sci. U.S.A. 84:8573-77), which contains the enhancer from cytomegalovirus immediate early gene fused to the promoter and tar region of HIV (nucleotides -69 to +78). Some of these plasmid vectors contain alternative enhancer:promoter elements derived from the cytomegalovirus immediate early gene (designated CMV) or the mouse metallothionein-I gene (Mt). These regulatory elements are linked to gene sequences encoding HIV proteins which are followed by a HpaI to HhaI (nucleotides 1569 to 1680) fragment of Adenovirus2 containing the Ela gene polyA addition site, followed by the BamHI to SphI fragment of pBR322, cloned into the plasmid Bluescribe plus (Strategene). The individual HIV proteins encoded by each of these plasmid vectors are indicated in Table I, and schematic diagrams of these plasmids are in FIG. 9.

TABLE I

HIV GENES ENCODED BY PLASMID VECTORS

Vector	HIV Genes				
	Gag+Pro	Tat	Rev	Env	Other
CmHiEnv5 (1104-(bl)				+	
CmHiTgfbEnv5 (1113-al)				+	
CmHiGag2Rre (1158-al)	+				
CmVGag2Rre (1159-al)	+				
CmHiRev (1132-cl)			+		
CmvRev (1152-al)			+		
CmHiTat (1132-bl)		+			
BsMtRev (1202-2)			+		
BsMtTAT (1203-1)		+			
CmHIVdelXmn (1133-al)		+	+	+	Vif,Vpr, Vpu
CmHIVdelKpnAvr(Gag2TRE) (1160-al)	+	+	+	+	Vpu

The plasmid CmHiEnv5 (1104-b1), has the NruI to HindIII fragement of pH3MPy, containing the Cytomegalovirus immediate early gene enhancer and HIV promoter and tar element, linked to nucleotides 5671-8572, AvaII to KpnI.

Plasmid CmHiTgfbEnv5 (1113-al) contains the NruI to PstI enhancer:promoter fragment of pH3MPy linked to chimeric TGF-β:HIV env gene; the simiam TGF-beta gene, providing the 5'untranslated region and signal peptide, is fused directly at the signal cleavage site of the HIV env gene, extending from nucleotide 5856 to the KpnI site at nucleotide 8572.

Plasmid CmHiGag2Rre (1158-al) contains the NruI to XbaI enhancer:promoter fragment of pH3MPy linked to HIV gag and pol coding sequences extending from the BssHII site at nucleotide 257 to the Asp718 site at nucleotide 3372. This is the same region of gag coding sequence contained in the recombinant vaccinia virus v-gag2, and includes the entire gag reading frame followed by about half of the pol reading frame. Also included in this region are sequences which encode the HIV protease and much but not all of the region encoding reverse transcriptase. An XbaI linker providing

translational termination codons was ligated to the Asp^{718} site. This is followed by a fragment of the HIV env gene, extending from BglII at 7178 to HindIII at 7698, containing the Rev responsive element. Plasmid CmvGag2Rre (1159-a1) is identical except that the NruI to XbaI cytomegalovirus enhancer and promoter fragment derived from the expression vector CDM8 (Invitrogen) was used.

Plasmid CmHiRev (1132-c1), contains the NruI to PstI enhancer:promoter fragment of pH3MPy upstream of an intronless HIV Rev gene, extending from the Bsu36I site at nucleotide 5500 to the KpnI site at nucleotide 8572, with nucleotides 5590-7935 deleted. Plasmid CmvRev (1152-a1) is identical, except that the NruI to XbaI cytomegalovirus enhancer and promoter fragment derived from the expression vector CDM8 (Invitrogen) was used.

Plasmid CmHiTat (1132-b1) contains the NruI to BglII enhancer:promoter fragment of pH3MPy upstream of an intronless HIV tat gene, extending from SalI site at nucleotide 5331 to the KpnI site at nucleotide 8572, with nucleotides 5590-7935 deleted.

Plasmid BsMtRev (1202-2) contains a 1.7 kilobase fragment of the mouse metallothionein-I gene extending from an EcoRI site at approximately position -1700 to the transcriptional start site, upstream of an intronless HIV rev gene, extending from the Bsu36I site at nucleotide 5500 to the KpnI site at nucleotide 8572, with nucleotides 5590-7935 deleted.

Plasmid BsMtTat (1203-1) contains a 1.7 kilobase fragment of the mouse metallothionein-I gene extending from an EcoRI site at approximately position -1700 to the transcriptional start site, upstream of an intronless HIV tat gene, extending from the SalI site at nucleotide 5331 to the KpnI site at nucleotide 8572.

Plasmid CmHIVdelXmn (1133-a1) contains the same hybrid CMV:HIV Enhancer:Promoter derived from the expression vector pH3MPy used in plasmids described above. This plasmid

incorporates HIV 5' leader RNA sequences to the XmnI site at nucleotide 384 just inside the N-terminus of the gag reading frame; contained within this segment is the splice donor site that is spliced to acceptor sites located upstream of many HIV genes, including those encoding the tat, rev and env proteins. The XmnI site at nucleotide 384 is joined through an XbaI linker (which contains a TAG translation termination codon, stopping translation of a gag N-terminal peptide after about 20 amino acid residues) to an XmnI at nucleotide 4034 near the C-terminus of the pol reading frame. HIV sequences then continue to the KpnI site at nucleotide 8572, through the region encoding both HIV regulatory and minor structural proteins including vif, vpr, tat, rev, vpu, and for the HIV env protein. This plasmid should be able to encode each of the HIV proteins listed above through splicing between the 5' splice donor site located just before the N-terminus of the gag gene and splice acceptor sites located upstream of the various protein coding reading frames.

The plasmid CmHIVdelKpnAvr(Gag2TRE) (1160-a1) contains hybrid CMV-HIV Enhancer:Promoter driving expression of the entire gag reading frame and the N-terminal portion of the pol reading frame (contained in the recombinant vaccinia virus v-gag2) directly joined in the 3' portion of the HIV genome containing sequences encoding the tat, rev and env proteins. In this plasmid the NruI to EcoRI Cytomegalovirus immediate early gene enhancer segment of expression vector P3MPy is linked to HIV promoter sequence begining at nucleotide -69; HIV leader RNA sequences continue through the gag gene and into the pol gene to the KpnI site at nucleotide 3372, joined to a polylinker which includes an NheI containing a TAG translation termination codon for the pol reading frame. This NheI site is joined to an AvrII site at nucleotide 5207 in the vpr reading frame. HIV sequences then continue to the KpnI site at nucleotide 8572. This vector contains all of the sequence elements believed

to important for the synthesis, splicing, cytoplasmic transport, translation, processing and function of HIV gag, protease, tat, rev, and env proteins. This plasmid should be able to encode HIV gag from unspliced mRNA and for each of the other HIV proteins listed above through splicing between the 5′ splice donor site located just before the N-terminus of the gag gene and splice acceptor sites located upstream of the various protein coding reading frames. It also includes the tar element located within the first sixty nucleotides of the HIV primary mRNA transcript which is required for tat transactivation from the HIV promoter, and the Rre (rev responsive element) located between nucleotides 7315 and 7559 which is required for rev catalyzed cytoplasmic localization of mRNAs encoding HIV structural proteins. Not contained in this plasmid vector are a central region of the HIV genome encoding the C-terminal half of the pol reading frame, including part of the reverse transcriptase protein and all of the integrase protein, all of the vif reading frame and the N-terminus of the vpr reading frame; also absent from this vector is most of the 5′ LTR and all of the 3′ LTR as well as most of the nef reading frame.

8.2. GENERATION OF RECOMBINANT-MADE HIV-1 PARTICLES IN STABLE CHO CELL TRANSFECTANTS

Plasmid vectors CmHIVdelKpnAvr(Gag2TRE) and CmHiEnv5 were transfected into dhfr- CHO cells. Transfected cell lines were selected by cotransfection with plasmid pSV2dhfr. CHO transfectants contained immunoreactive mature gag and env proteins, secreted in a particulate form with sedimentation properties similar to those of the HIV virion and the recombinant-made HIV-1 particles produced as described in Section 6., et seq., supra.

8.2.1. TRANSFECTION AND SELECTION OF CELLS

CHO cells (dhfr-) were transfected following growth in

Ham's F12 nutrient mixture (without hypoxanthine) supplemented with 10% fetal bovine serum and 150 μg/ml proline in 60 mm tissue culture dishes to 50% confluency, transfer to serum free medium, incubation for 5 hours with a mixture of Lipofectin (BRL), the HIV expression plasmids CmHIVdelKpnAvr(Gag2TRE) (1160-a1) and CmHiEnv5 (1104-b1), and the selectable plasmid pSV2dhfr, removal of the Lipofectin:DNA mixture and transfer back to medium contianing serum. Two or three days post-transfection the cultures were transferred to selective medium, DMEM plus 10% FBS and proline. Two days later the cultures were trypsinized, and aliquots containing approximately 0.08% and 0.40% of the cells (estimated to contain approximately 1 and 5×10^3 transfected cells, respectively) were transferred to 96 well tissue culture wells. Aliquots containing 7.8% of the cells were also transferred to 6 well tissue culture dishes.

After 13 days of further culture in selective medium, a set of 6 well plates were fixed and cells were immunostained for expression of HIV proteins by incubation with serum from an AIDS patient (TriMar), followed by horseradish peroxidase conjugated goat anti-human antibody, and a peroxidase substrate (3-amino-9-ethyl-carbazole). There were 15-20 colonies of selected cells per well; approximately 10% of these colonies were found to contain HIV proteins by the immunostaining assay. This analysis indicated that only about 10 to 15 wells per 96 well plate seeded with the lower number of cells should contain a colony of transfected cells, and that a fraction of these would likely express HIV proteins.

Tissue culture media from individual wells of the 96 well plates were then collected and assayed by a gag protein antigen EIA for secreted gag protein (Section 6.1.2, supra). A number of candidate wells were identified by this assay. Twelve potentially positive wells, four wells derived from each of three independent transfections, were then chosen

for expansion. Visual insepection of positive wells at this point revealed that some wells were essentially confluent, others had only small colonies of growing cells, and others apparently contained no viable cells. Cells from all three classes of potentially positive wells were expanded; wells were trypsinized and cells transferred to 6 well dishes for expansion.

In addition, a small fraction of the cells from each candidate cell line was seeded into duplicate wells of a 24 well plate. After five days of growth, wild type HeLa cells or HeLa T4 cells (transfected with and expressing the CD4 protein) were added to each well. The following day, gag expression and env function was assayed by a focal immunoassay. Briefly, cells were fixed and incubated with a human anti-HIV serum, followed by a horseradish peroxidase linked goat anti-human antibody, and a peroxidase substrate (3-amino-9-ethyl-carbazole). Four cell lines, falling into two classses, were positive by this assay. Two lines (3010-C1 and 3010-C6) had only a small number of cells, but these stained very intensely and efficiently formed giant syncitia with HeLa T4 cells, but not with wild type HeLa cells. Two other lines (3010-C5 and 3010-C13) had many positive cells, but these stained less strongly and only a fraction formed syncitia with HeLa T4 cells. Cells from other lines failed to stain. The antibody staining of the positive cells suggests that they are synthesizing gag proteins, and the formation of syncitia with HeLa T4 cells demonstrates that they have functional gp120 and gp41 envelope proteins on their cell surface.

After further expansion of each of the candidate lines, cell lysates were analyzed by Western blot for HIV protein synthesis. Cells in 60 mm or 100 mm tissue culture dishes were washed twice with PBS and collected directly into 300 μl or 750 μl of Laemmli sample buffer. Afer boiling, the total cellular protein in the sample was resolved by electrophoresis on a 10% polyyacrylamide gel or an 8-16%

gradient polyacrylamide gel. Aliquots of HIV, CEM cells infected with HIV, and BSC-40 cells infected with vaccinia recombinants v-gag1, v-gag2, or v-env5 were included as controls. The contents of the gel were electro-transferred to a sheet of nitrocellulose filter. The filter was reacted with either AIDS patient serum or serum from a rabbit immunized with gp160 derived from v-env5 infected cells, extensively washed, reacted with 125-I labeled Protein A, and extensively washed again. The products were then visualized by autoradiography with X-ray film.

The four positive lines identified in the previous assay, were also found to be positive for gag proteins. 3010-C1 and 3010-C6 lysates contained several-fold more gag than did 3010-C5 and 3010-C13 lysates. Lines that were negative in the previous assay were also negative for gag by Western blot. Most of the immunoreactive material co-migrated with the p55 precursor and as a slighter smaller species not corresponding to a major product in HIV or in cells infected with HIV or v-gag1; only very minor amounts of mature p24 and p17 gag proteins were detectable in cell lysates. This analysis also demonstrated the presence of gp160, gp120 and gp41 envelope proteins in cell lysates of the positive cell lines.

8.2.2. CHARACTERIZATION OF RECOMBINANT-MADE HIV-1 PARTICLES

To demonstrate that the gag and env proteins synthesized in these cells were assembled into virus-like particles and secreted, culture supernatants were collected and cleared by low speed centrifugation. The particle fraction was collected by centrifugation (either 32,000 rpm in the SW55 rotor, 27000 rpm in the SW41 rotor, or 19000 rpm in the Type 19 rotor). Western blot analysis demonstrated that this material contains both gag and env proteins. The primary gag proteins detected in the particles are the mature p24 and p17 species; smaller amounts of the p55 and p40

precursors could also be detected. In contrast, precursor species accounted for most of the material in cell lysates. Gp160, gp120, and gp41 envelope proteins were all detected in the particles.

Comparison of Western blots suggests that only a small fraction of the gag and env proteins contained in the cells are secreted as particles. Quantitative gag antigen EIA analysis of multiple 3010-C6 cell-derived particle preparations suggested yields of 3-17 ng gag per ml of culture medium. Particles from the 3010-C6 cell line were also analyzed by sedimentation into a sucrose gradient (2 hours at 50,000 rpm in the SW55 rotor, 15-60% sucrose). As shown in FIG. 10, both gag antigen EIA and Western blot assay of fractions from the gradient showed that the particles banded in a single peak at approximately 35-40% sucrose.

8.3. ALTERNATIVE STRATEGIES FOR EXPRESSION OF ENV AND GAG PROTEINS AND GENERATION OF RECOMBINANT-MADE HIV-1 AND PARTICLES IN MAMMALIAN CELLS

In this section, a number of variations representing alternative strategies for the expression of HIV proteins and recombinant-made HIV particles in transfected cells are described. Possible variations include, but are not limited to, (1) the transfection of complex plasmid vectors containing multiple HIV structural and/or regulatory genes, (2) the co-transfection of multiple plasmid vectors containing different HIV genes, (3) the use of both constituitive and regulatable enhancer/promoter elements to drive the expression of HIV proteins, (4) the use of regulated expression of HIV regulatory proteins including tat and/or rev to indirectly control the expression of HIV gag and env structural proteins, and (5) the expression of HIV proteins in different cell types. Such variations may be useful in optimizing various parameters of the system, and provide for the independent manipulation of the protein

components of the particles and the levels of expression in different cell types.

Plasmids and combinations of plasmids were tested by transient transfection into HeLa (and in a few cases BSC-40 and Vero) cells by standard calcium phosphate transfection procedures. Products were analyzed by polyacrylamide gel electrophoresis of total cell lysates or of recombinant-made HIV particles collected from culture medium by high speed centrifugation, electro-transfer to nitrocellulose filters, and probing with specific anti-sera. For analysis of env proteins, the filter was probed with 125-I labeled monoclonal antibody 110-4 (Section 6.1.2., supra), which binds an epitope in the V3 region of gp160 and gp120; for analysis of gag proteins the filter was probed with AIDS patient serum (TriMar) followed by 125-I labeled Protein A. The products were then visualized by autoradiography with X-ray film.

8.3.1. EXPRESSION OF HIV PROTEINS IN HeLa CELLS CAN BE OBTAINED FROM MANY DIFFERENT PLASMIDS AND COMBINATIONS OF PLASMIDS

Expression of HIV gag and env proteins can be obtained by transfection of complex plasmids encoding multiple HIV proteins, including both structural and regulatory proteins; alternatively, multiple plasmids encoding different HIV proteins can be transfected together.

Transfection into HeLa cells of plasmids coding individually for env (CmHiTgfbEnv5 or CmHiEnv5), or for gag (CmHiGag2Rre), in combination with plasmids encoding tat and rev proteins results in the expression of immunoreactive gp160 and pg120 HIV envelope proteins or in the expression of immunoreactive HIV gag related proteins including the p55 primary translation product, p47 and p39 processing intermediates, and p24 and p17 mature gag proteins.

Alternatively, transfection into HeLa cells of plasmid vectors (CmHIVdelXmn or CmHIVdelKpnAvr(Gag2TRE)), which

contains in a single transcriptional unit the functional coding sequences for the both the tat and rev regulatory proteins and the env, or both env and gag, structural protein(s) also results in the synthesis of both precursor and mature env or gag and env proteins. Co-transfection of CmHIVdelXmn with CmHiGag2Rre or CmVGag2Rre also results in the synthesis of both gag and env proteins. Each of the plasmids or combinations CmHIVdelKpnAvr(Gag2TRE) (1160-al), CmHIVdelXmn (1133-al) + CmHiGag2Rre (1158-al), and CmHIVdelXmn (1133-al) + CmvGag2Rre (1159-al) also results in the secretion of recombinant-made HIV particles into the culture medium.

Thus, the expression of gag and env structural proteins and the production of recombinant-made HIV particles using this system may be achieved by multiple routes. Comparisons of the levels of expression achieved with different plasmid combinations are shown in Table II, below, demonstrating that different combinations may be optimal for the expression of different proteins. For example, env appears to be more efficiently expressed when co-transfected with plasmids coding separately for tat and rev proteins. On the other hand, gag may be more efficiently expressed from a plasmid such as CmHIVdelKpnAvr(Gag2TRE) (1160-al), which contains in a single transcriptional unit the functional coding sequences for both regulatory proteins. Intermediate levels of both proteins were obtained by co-transfection of the complex plasmid CmHIVdelKpnAvr(Gag2TRE) (1160-al) and a separate env coding plasmid.

TABLE II

RELATIVE LEVELS OF EXPRESSION OF env AND gag PROTEINS OBTAINED USING DIFFERENT COMBINATIONS OF PLASMIDS

Plasmids	env	gag
CmHiEnv5 + CmHiTat + CmHiRev	++++	-
CmHiGag2Rre + CmHiTat + CmHiRev	-	++
CmvGag2 + CmvRev	-	+
CmHIVdelXmn	+	-
CmHiGag2Rre + CmHIVdelXmn	+	++++
CmHIVdelKpnAvr(Gag2TRE)	+	++++
CmHiEnv5 + CmHIVdelKpnAvr(Gag2TRE)	+++	+++

Relative expression levels of gag and env obtained after transfection with various combinations of plasmids are indicated in arbitary units; gag and env expression levels were determined independently, and are not necessarily represented by the same scale.

In these examples, rev is important for the efficient cytoplasmic localization of mRNAs encoding HIV structural proteins, and is dependent on a cis-acting regulatory element, termed the rev responsive element (Rre), located within the portion of the HIV env gene encoding the N-terminal portion of the gp41 molecule, which was introduced into the gag plasmids as a BglII to HindIII fragment from the envelope gene, and may be intrisinically necessary for efficient expression of HIV gag and env proteins. The tat protein is important for enhancing transcription initiated from the HIV promoter by its interaction with a cis-acting regulatory element, called tar, located within the first 75 nucleotides of HIV RNA transcripts. The tar gene is present in the CmHi hybrid enhancer element utilized by CmHiGag2Rre, CmHiTgfbEnv5, and CmHiRev.

Expression of gag or env can be made independent of tat by co-transfection of plasmids such as CmvGag2Rre and CmvRev, since these plasmids do not contain the tar region. However, gag expression from transfection of CmvGag2Rre + CmvRev is lower than from transfection of CmHiGag2Rre + CmHiTat - CmHiRev. Since the enhancer:promoter of the cytomegalovirus immediate early gene is known to be a very strong transcriptional element, this demonstrates that the combination of the CMV:HIV Enhancer:Promoter element plus the HIV tat gene may be an especially strong expression system. Similar observations were also made in the context of env protein expression. Hybrids between the other enhancer elements and the HIV promoter/tar element may have other useful properties.

These results demonstrate that different HIV structural proteins may differ in their requirements for HIV regulatory proteins or other factors to realize maximal expression, and that by choosing appropriate plasmids or combinations of plasmids the ratio of gag to env proteins may be controlled.

These results also indicate that plasmids encoding different HIV proteins may be independently introduced into cells. As one example, a cell line producing recombinant-made HIV particles may be further transfected with a plasmid encoding a minor HIV protein such as vpu or vif to alter the production levels and/or properties of the recombinant-made HIV particles. As another example, cell lines transfected with a plasmid encoding tat, rev and gag proteins, which therefore produce particles containing only gag proteins, could be selected and subsequently transfected with different env coding plasmids to generate particles containing env proteins from different strains or having other strutural alterations. For example, upon comparing the expression of env proteins from genes containing either the natural signal sequence of the HIV env genes (CMHIEnv5) or a fusion between the signal sequence of simian TGF-beta-1 and the N-terminus of mature gp160 (CmHiTgfbEnv5), equally

efficient expression was observed.

8.3.2. REGULATION OF HIV env PROTEIN EXPRESSION BY THE USE OF REGULATABLE PROMOTERS

In another embodiment, dhfr- CHO cells were transfected with the plasmid combination CmHiTgfbEnv5 + CmHiTat + CmHiRev, along with the selectable plasmid pSV2dhfr. Initial examination of cell lines selected in this experiment did not reveal detectable levels of HIV env expression. Cells were then further selected by growth in increasing levels of methotrexate for amplification of the transfected selectable pSV2dhfr plasmid and co-amplification of the HIV tat, rev and env coding plasmids in the hope that this would select for amplification of low levels of HIV env expression. One cell line was identified in which HIV env proteins were being synthesized. However, selection for further amplification of the dhfr gene and the co-transfected HIV protein encoding plasmids by growth in progressively higher concentrations of methotrexate did not lead to increased env protein expression. Rather, continued selection was required to maintain env protein expression levels, as envelope protein expression levels decreased in cells grown without escalating selection. Cells expressing higher levels of env proteins also seemed to grow more slowly and to a lower density than cells lines that had reverted to lower levels of env protein expression. These results suggest that envelope protein expression is toxic in these cells, and may therefore represent a limitation on the attainable levels of env protein expression.

This limitation may be overcome by the use of plasmids utilizing regulatable promoters controlling the expression of HIV proteins. Such plasmids can be transfected into cells which would initially be grown in the uninduced state, then be induced to high levels of HIV protein expression to allow short-term high levels of production before the onset of the toxic effect.

As an example of such an approach, plasmids individually encoding tat and rev proteins under the control of the metal regulated mouse metallothionein-I promoter were constructed. Co-transfections of HeLa cells with the plasmid combinations, (a) CmHiTgfbEnv5 + CmHiTat + CmHiRev, (b) CmHiTgfbEnv5 + BsMtTat + CmHiRev, and (c) CmHiTgfbEnv5 + CmHiTat + BsMtRev, followed by Western blot analysis of total cellular lysates were all shown to produce immunoreactive gp160 and gp120 HIV envelope proteins (FIG. 11). Transfection with CmHiTgfbEnv5 + CmHiTat + CmHiRev generated the highest level of env protein expression. More significantly, in the transfections utilizing the Mt promoter, expression levels were found to be induced several-fold by the addition of zinc to the tissue culture medium about a day prior to harvest. (See FIG. 11).

These results demonstrate that regulatable promoters can be used to modulate the expression of HIV proteins, and that the expression of HIV structural proteins can be indirectly regulated by modulation of the HIV tat and rev regulatory proteins. Other examples of this approach include the use of regulatable promoters to control expression from complex plasmids encoding multiple HIV proteins, or the construction of novel inducible regulatory elements, for example, such as a hybrid Mt:Hi enhancer promoter containing the metal regulatory elements of the metallothionein promoter linked to the promoter and tar region of HIV.

8.3.3. EXPRESSION OF HIV PROTEINS IN BSC-40 AND VERO CELLS

In this example various plasmid combinations encoding HIV tat, rev, env and gag proteins, including CmHiGag2Rre + CmHiEnv5 + CmHiTat + CmHiRev together, and CmHIVdelKpnAvr(Gag2TRE), were transfected into HeLa, BSC-40 and Vero cells. The basic pattern of expression of gag and env, both with respect to the products made and expression efficiency are similar in all three cell lines. However, overall expression levels are highest in HeLa cells,

approximately 3-fold lower in BSC-40 cells, and another 5-fold lower in Vero cells.

These results, along with those described above with CHO cells, demonstrate that a variety of cell lines, derived from different species and different organs can be used to express HIV proteins, so that cells with particular desired characteristics can be chosen for particular applications.

9. EXAMPLE: GENERATION OF RECOMBINANT HIV-1 PARTICLES HAVING MODIFIED STRUCTURAL CHARACTERISTICS USING RECOMBINANT VACCINIA VIRUS EXPRESSING TRUNCATED FORMS OF HIV-1 ENVELOPE ANTIGENS

Two recombinant vaccinia viruses which direct the expression of truncated HIV-1 gp160 in infected BSC-40 cells are described below. These recombinant vaccinia viruses may be used as vectors, in conjunction with v-gag2 (Section 6., et seq., supra) or other core antigen-encoding vectors, for generating recombinant HIV-1 particles which may have enhanced anti-viral and/or immunogenic properties using the system described in Section 6., supra.

9.1. RECOMBINANT VACCINIA VIRUS V-ED2

A recombinant plasmid was constructed which contained the HIV-1 env encoding sequence from nucleotide numbers 5705 to 8068 inserted into vaccinia recombination vector pGS62 (copending United States Patent Application Serial No. 07/593,401 filed October 5, 1990) at the BamHI site downstream from the 7.5K promoter. The HIV-1 sequence was derived as a 2.36 Kbp BamHI fragment from plasmid pv-env5 (copending United States Patent Application Serial No. 07/593,401, filed October 5, 1990). The fragment contained the entire coding sequence of gp120 and the N-terminal 241 amino acids of gp41, including 49 amino acid residues of the cytoplasmic region of gp41 at the C-terminus of the proposed transmembrane sequence. The chimeric gene containing the 7.5K promoter and the HIV-1 env sequences was inserted into the vaccinia virus thymidine kinase gene according to

procedures described in copending United States Patent Application Serial No. 07/593,401, filed October 5, 1990.

The resultant recombinant virus v-ED2 directs the expression of a truncated gp160 which is cleaved into gp120 and gp41 as efficiently as wild-type gp160 (FIG. 12). HeLa $CD4^+$ cells infected with v-ED2 formed syncytia (multinucleated giant cells, characteristic of HIV-1 infection) more readily than v-env5, which expresses a full-length gp160 envelope glycoprotein precursor. These results indicate that the envelope glycoproteins produced by v-ED2 may be functionally more active or more efficiently presented than "wild-type" envelope glycoproteins.

9.2. RECOMBINANT VACCINIA VIRUS v-ENV5DCT

Recombinant virus v-ENV5DCT was constructed to contain the entire env coding sequence of HIV-1v (BRU isolate; Wain-Hobson et al., 1985, Cell 40:9317), except for the C-terminal 13 amino acids of gp41. The deletion mutation was introduced by an oligonucleotide mutagenesis procedure as described (Kunkel et al., 1987, Meth. In Enzymol. 154:367-382). The chimeric gene containing the 7.5K promoter and the mutated HIV-1 env sequences was inserted into the vaccinia virus thymidine kinase gene according to procedures described in copending United States Patent Application Serial No. 07/593,401, filed October 5,1990.

10. EXAMPLE: GENERATION OF RECOMBINANT HIV-1 PARTICLES HAVING MODIFIED STRUCTURAL CHARACTERISTICS USING RECOMBINANT VACCINIA VIRUSES EXPRESSING UNPROCESSED GP160

Two recombinant vaccinia viruses which direct the expression of a gp160 precursor envelope glycoprotein having mutations in the proteolytic cleavage site(s) between gp120 and gp41 are described herein. These recombinant vaccinia viruses may be used as vectors in conjunction with v-gag2 or other core-antigen-encoding vectors for generating recombinant HIV-1 particles which may have enhanced

antiviral and/or immunogenic properties using the system described in Section 6., supra.

10.1. RECOMBINANT VACCINIA VIRUS v-160NC

A recombinant plasmid (pv-160NC) was constructed that contained a mutated gp160-coding sequence from nucleotide numbers 5803 to 8495 inserted downstream from the 7.5K promoter in vaccinia recombination vector pGS62. The mutations were introduced by oligonucleotide-directed mutagenesis essentially as described (Kunkel et al., 1987 (Meth. Enzymol. 154:367-382). The mutated sequences are shown below ("†" represents cleavage site between gp120 and gp41):

HIV-1 BR env			gp120			†	gp41	
		Gln	Arg	Glu	Lys	Arg	†	Ala
"wild-type"	...CAG	AGA	GAA	AAA	AGA	†	GCA...	
v-160NC	...CAG	ATA	GAA	GAA	TTC	†	GCA...	
		Gln	Ile	Glu	Glu	Phe	†	Ala

The chimeric gene was inserted into the vaccinia virus genome at the thymidine kinase gene by in vivo recombination as described in copending United States Patent Application Serial No. 07/593,401, Filed October 5, 1990. The resulting recombinant virus directs the expression of a gp160 precursor envelope glycoprotein that is not cleaved into gp120 and gp41.

10.2. RECOMBINANT VACCINIA VIRUS v-11K160NC

A recombinant vaccinia virus containing the same mutated HIV-1 env gene as v-160NC (Section 10.1., supra), but under the control of vaccinia virus 11K promoter was constructed. A 2.26 Kbp BamHI fragment was excised from plasmid pv-160NC and was inserted into a derivative of vaccinia recombination vector pSC10 (Chakrabarti et al., 1985, Mol. Cell. Biol.

5:3403-3409) at the EcoRI site downstream from the 11K promoter. This generated a chimeric gene containing the vaccinia virus 11K promoter and the entire coding sequence for the mutated gp160. The chimeric gene was inserted into the vaccinia virus genome at the thymidine kinase gene. The resultant recombinant virus directs high level production of mutant HIV-1 gp160 in infected cells.

11. EXAMPLE: RECOMBINANT VACCINIA VIRUS EXPRESSING HIV-1 vpu GENE

The assembly and production of recombinant-made HIV-1 particles requires no HIV-1-specific proteins other than the co-expression of HIV-1 envelope and core antigens. However, other factors, both of viral and cellular origins, may participate and enhance this process. One such factor is a viral protein encoded by vpu. This protein is a non-glycosylated polypeptide of 16 Kd apparently associated with the inner surface of the cytoplasmic membrane. Although vpu is not required for particle formation, mutations in vpu result in a decrease in virions released from infected cells (Terwilliger et al, 1989, Proc. Natl. Acad. Sci. U.S.A. 86:5163-5167; Strebel et al., 1989, J. Virol. 63:3487-3791). The mechanism of vpu action is not known and its role in particle formation in a recombinant system, such as the one described here, has not been defined. It is anticipated that accessory molecules, such as vpu, may play a role in facilitating the process of virion assembly and/or release. The following example describes a system with which the potential role of such accessory molecules could be examined and their utility in recombinant particle production demonstrated.

A recombinant vaccinia virus was constructed that contained the entire HIV-1 vpu-coding sequence. A 290-bp fragment of HIV-1 cDNA from nucleotide numbers 5636-5927 was inserted into vaccinia recombination vector pGW62 between the SmaI and EcoRI sites downstream from the 7.5K promoter.

This fragment contains the coding sequence of vpu as well as 7 bp of 5'- and 37 bp of 3'-untranslated sequences. The chimeric gene was then inserted into the vaccinia virus genome by in vivo recombination. The effect of vpu on recombinant particle formation and release can be demonstrated in cells co-infected with v-vpu and v-G2E5 as described in Section 7, et seq., supra.

12. EXAMPLE: ANTIVIRAL EFFECT OF RECOMBINANT-MADE HIV-1 PARTICLES

This example demonstrates the ability of the recombinant-made HIV-1 particles of the invention to reduce or abrogate the infectivity of $CD4^+$ lymphocytes by HIV in vitro. The results indicate that recombinant-made HIV-1 particles effectively inhibit HIV-1 infection in a dose-dependent manner.

12.1. INFECTIVITY ASSAY

T-lymphoblastoid cells (CEM) were seeded into 24 well culture plates at $4x10^4$ cells/well in 0.4 ml culture media (RPMI-1640 supplimented with 10% Fetal Calf Serum). Duplicate wells then received varying amounts (see TABLE 1) of recombinant-made HIV-1 particles in 0.4 ml media. The added particles were quantitated by the equivalent p24 gag concentration determined by EIA (Section 6.1.2., supra). Control wells received no particles but were corrected for volume by the addition of 0.4 ml media. Cells and particles were allowed to incubate at 37°C for 3 hours prior to addition of HIV. A set of duplicate wells corresponding to each of the various particle concentrations received (1) no HIV input, (2) low virus input of 40 $TCID_{50}$ (tissue culture infectious dose units 50) corresponding to 5pg p24 gag, or (3) high virus input of 400 $TCID_{50}$, corresponding to 50pg p24. At day three post-infection, the culture media was withdrawn from the wells and replaced with fresh media containing the appropriate original concentration of

particles. At day five and then day six post-infection, aliquots of cells were collected from all wells and assayed for infectivity by determining intracellular expression of HIV antigens using an indirect immunofluorescence assay employing human polyclonal anti-HIV sera as primary antisera, followed by goat anti-human IgG fraction conjugated to fluorescene as secondary sera.

12.2. RESULTS

The infectivity assay results presented in TABLE 1, below, indicate that recombinant-made HIV-1 particles can block the infectivity of CEM cells by HIV virions in a dose dependent fashion. It can also be concluded that the recombinant particles themselves are non-infectious and do not replicate in the cell, since no intracellular fluorescence was detected in cells incubated with a high concentration of recombinant-made HIV-1 particles (FIG. 13, Panel A). Additionally, whereas multiple syncytia were observed in cell cultures receiving either of the HIV inputs (FIG. 13, Panel C), recombinant particles did not induce any syncytia formation.

TABLE III

INHIBITION OF HIV VIRUS INFECTIVITY
BY RECOMBINANT-MADE HIV-1 PARTICLES

CEM CELLS	RECOMBINANT-MADE HIV-1 PARTICLES (FOLD EXCESS)[1]	VIRUS[2] ($TCID_{50}$)	FLUORESCENCE % DAY 5	DAY 6
+	0	0	0	0
+	60	0	0	0
+	600	0	0	0
+	0	40	20	>90
+	60	40	10-20	>90
+	600	40	<1	10
+	0	400	90	>90
+	60	400	60-80	>90
+	600	400	30	>90

[1] Fold excess of particles to virus was in relation to equivalent amount of p24 protein.

[2] Human Immunodeficiency Virus Type 1 (HIV-1), strain LAV-1

13. EXAMPLE: RECOMBINANT-MADE HIV-1 PARTICLES INHIBIT HIV-1 INFECTION OF CULTURED PERIPHERAL BLOOD LYMPHOCYTES ISOLATED FROM HIV-1 SEROPOSITIVE DONOR

The example presented in Section 12., supra demonstrates that recombinant-made HIV-1 particles inhibited HIV-1 infection of T-lymphoblastoid cells (CEM) in vitro in a dose dependent manner. The following example confirms that the recombinant-made HIV-1 particles have antiviral effect using a cultured peripheral blood lymphocyte (PBL) system. The PBLs used in this system are isolated from HIV-1 infected seropositive donors, and the frequency of infected cells within the isolated PBL fraction ranges from 0.04% - 1.3% (Psallidopoulos, M.C. et al., 1989, J. Virol. 631:4626-4644). During culture, the virus infection spreads to the uninfected $CD4^+$ cells by cell-free and cell-cell

transmission. Recent results from other groups (Daar, E.S., et al. 1990 Proc. Natl. Acad. Sci. U.S.A. 87:6574-6578) suggested that laboratory isolates of HIV-1 responded differently than primary patient isolates to neutralization and to the effects of antiviral agents, such as soluble CD4. Therefore, using the cultured peripheral blood lymphocytes system to evaluate the antiviral effects of recombinant-made HIV-1 particles may be a more biologically relevant system than the CEM cell system described in Section 12., supra.

13.1. INFECTIVITY ASSAY

Peripheral blood lymphocytes (PBLs) were harvested from heparinized blood samples of HIV-1 seropositive donors by fractionation of the buffy coat material over a Hypaque-ficoll cushion. The cells were then incubated in culture media (RPMI-1640 supplemented with 10% Human Serum), and the $CD8^+$ lymphocytes were depleted by treatment with CD8, CD16, and CD20 specific monoclonal antibodies for 1 hr at 37°C followed by the addition of rabbit complement for 1 hr at 4°C (Zarling, J.M. et al., 1990, Nature (London) 347: 92-95). The resulting cell preparation consisted mainly of $CD4^+$ lymphocytes, B lymphocytes and macrophages. The cells were seeded into 24 well plates at 1M cells/well in 0.5 ml culture media. Duplicate wells received varying concentrations (see Tables IV and V) of either recombinant-made HIV-1 particles, or psoralen/U.V.-inactivated HIV-1 virions. All wells were then supplemented with 0.5 ml of culture media containing MAb G19.4 reactive with T cell CD3 antigen (Hoxie, J.A., et al. 1986 Science 234:1123-1127) at lug/ml final concentration as well as interleuken 2 (IL-2) at a final concentration of 5%. Activation of T cells with a soluble CD3 MAb has been shown to induce the replication of latent HIV-1 virus (Zarling, J.M. et al., 1990, Nature (London) 347: 92-95).

Four or five days following activation (donors Z29,Z30 or donors Z31,Z39 respectively), the culture media was

withdrawn from the wells and the cells were washed with PBS supplemented with 10% human serum and replaced with fresh culture media. Identical samples were either allowed to incubate with the fresh media alone (see below) or were again supplemented with the appropriate concentrations of recombinant-made HIV-1 particles or inactivated HIV-1 virus. At certain intervals following the secondary addition of the particles or inactivated virus, as indicated in Table IV, samples of culture media were harvested from the wells and analyzed for $p24^{gag}$ content by specific EIA (Section 6.1.2., supra). Additionally, cells were concomitantly collected and examined for HIV-1 protein expression by indirect fluorescence using a mixture of monoclononal antibodies reacting with gp120 and $p24^{gag}$.

Cells that had the recombinant-made particles or inactivated virus washed out on day four or five, were passaged on day 8 into new wells containing immobilized anti-CD3 MAb to induce continued replication of the cells. On day 11, samples of culture supernatants were assayed for $p24^{gag}$ production by EIA (see Table V). This portion of the experiment was designed to assess whether the inhibition of virus production detected following treatment with the recombinant-made HIV-1 particles was a reversible phenomenon.

13.2. RESULTS

The results of the infectivity assays are presented in Tables IV and V. The recombinant-made HIV-1 particles inhibited the spread of virus infection through the culture in samples derived from four different seropositve patients. This inhibition was dose dependent and was detectable at 0.1 μg/ml eqivalent of $p24^{gag}$ protein in samples treated with recombinant-made HIV-1 particles. Although the psoralen/U.V. inactivated virus similarly inhibited the spread of virus infection in the cultures, in some instances (Table IV, donor Z29, day 6 samples), 10-fold more

inactivated virus was needed compared to the recombinant-made HIV-1 particles.

The persistent inhibition of $p24^{gag}$ production by cells that had washed free of the recombinant-made HIV-1 particles or the inactivated virus on day four (Table V) suggested that the removal of the particles was not sufficient to reverse the phenomenon. Interestingly, the data also suggested that the recombinant-made HIV-1 particles were more efficacious in this inhibition than the inactivated HIV-1 virions (Table V, both donors).

TABLE IV

INHIBITION OF HIV-1 VIRUS INFECTIVITY
BY RECOMBINANT-MADE HIV-1 PARTICLES

DONOR	TREATMENT	DOSE(μg/ml)[1]	PERCENT INHIBITION OF $p24^{gag}$ PRODUCTION
			day 9
Z31	Particles	1	86
		0.1	18
		0.01	0
Z39	Particles	1	99
		0.1	5
		0.01	0
			day 6
Z29	Particles	10	96
		1	97
		0.1	73
		0.01	15
	Inactivated HIV	10	90
		1	78
		0.1	0
Z30	Particles	10	86
		1	84
		0.1	22
		0.01	0
	Inactivated HIV	10	84
		1	78
		0.1	0

[1]Concentration of recombinant-made HIV-1 particles and inactivated virus are listed with respect to the $p24^{gag}$ concentration. The relative envelope glycoprotein content of these preparations is 5-10% that of $p24^{gag}$.

TABLE V

INHIBITION OF HIV INFECTION BY THE RECOMBINANT-MADE HIV-1 PARTICLES IS IRREVERSIBLE

DONOR	TREATMENT	DOSE (μg/ml)[1]	PERCENT INHIBITION OF $p24^{gag}$ PRODUCTION[2]
Z29	Particles	10	>79
		1	>51
		0.1	0
		0.01	0
	Inactivated HIV	10	>44
		1	0
		0.1	0
Z30	Particles	10	99
		1	91
		0.1	52
		0.01	4
	Inactivated HIV	10	50
		1	15
		0.1	1

[1]Concentrations of treatments are as described in Table IV.

[2]Inhibition of $p24^{gag}$ production was assayed on day 11 post-activation. The cells were washed on day 4 and allowed to recover in the absence of treatment. To insure cell growth the cells were cultured in the presence of 5% Il-2, and exposed to immobilized CD3 MAb on day 8.

14. EXAMPLE: IMMUNOGENICITY OF RECOMBINANT-MADE HIV-1 PARTICLES IN VIVO

The experiments described below were conducted to evaluate the in vivo immunogenicity of nonreplicating recombinant-made HIV-1 particles using a small animal model. Rabbits were chosen for these studies since previous reports indicated the presence of neutralizing antibodies in rabbits immunized with various forms of HIV-based immunogens.

The humoral immune response of each immunized rabbit was evaluated by ELISA and Western Blot analysis. The ELISA allowed the measurement of the overall HIV specific antibody titer, while the western blot analysis elucidated antibody reactivity with individual viral proteins. In addition, the cellular immune response elicited in two of the immunized rabbits was characterized. Results of these experiments confirmed the ability of the recombinant made HIV-1 particles to generate HIV-specific immune responses in immunized animals.

14.1. IMMUNOGENICITY ASSAY

Two New Zealand white female rabbits were immunized with either recombinant-made HIV-1 particles (Section 6., supra) or psoralen inactivated HIV-1 virus. The amounts of immunogens used were normalized according to their relative p24 gag protein concentrations as determined by capture EIA (Section 6.1.2., supra). The immunization schedule is set forth in Table VI, below.

For the primary immunization (1°), the immunogens were formulated with Complete Freunds adjuvent at 1:1 ratio and administered by intramuscular injections at amounts equivalent to 120ug p24 gag and 4-6ug gp120 env proteins. Five weeks later, the rabbits received secondary immunizations (2°, boost) by the subcutaneous route of material formulated in Incomplete Freunds adjuvent also at a 1:1 ratio. The amount of immunogen used in the 2° immunization was equivalent to 190ug p24 and 10-20ug gp120

proteins (rabbit 238 and 239), or 100 μg p24 and 5 to 10 μg gp120 protein (rabbits 241 and 243) (see Table VI). Tertiary immunizations were conducted only on recombinant-made HIV-1 particle immunized rabbits (see Table VI).

Sera collected from animals at various time intervals were assayed for anti-HIV reactivity by ELISA using immobilized whole disrupted virus or immobilized purified gp120 and/or Western blot analysis. Sera were also collected from each animal one week prior to the start of the experiment and used as preimmunization controls. For Western blot analysis, purified LAV-1 virus was solubilized in SDS-PAGE sample buffer, and fractionated by electrophoresis in a acrylamide gradient (7%-15%) slab gel. The fractionated viral proteins were transferred to a nitrocellulose filter using standard techniques. The filter was subsequently divided into identical strips and used in the Western blot analysis. Briefly, following blocking of the strips for 3 hours with blocking buffer (3% dried milk in PBS, BLOTTO) at 22°C, each strip was incubated with a 1:50 dilution, in BLOTTO 0.2% NP40, of specific rabbit serum sample overnight at 4°C. The strips were then washed extensively, and incubated with ^{125}I Protein A (ICN Radiochemicals) in BLOTTO 0.2% NP40 for 2 hours at 22°C. Protein bands were visualized by autoradiography. The sera were also tested for the presence of neutralizing antibodies that reduce the level of $p24^{gag}$ production, an indicator of virus production and release.

Presence of antibodies that neutralize the LAV-BRU isolate in the sera of the immunized rabbits were detected by using a <u>in vitro</u> CEM cell infectivity assay. Sera were heat inactivated and then serially diluted in culture medium (RPMI-1640 supplemented with 10% Fetal calf serum). Equal volumes of diluted serum and 30 $TCID_{50}$ of virus inoculum were mixed and incubated at 37° for 45 min. The challenged virus preparation (0.1ml) was added to 0.1ml cultures of CEM cells ($2x10^4$ cells) in 96-well microtiter plates and

incubated at 37°C with occasional mixing. After 1 hour incubation, the virus-serum mixture was removed from the wells and replaced with culture medium containing the original rabbit serum at the appropriate diultion. After 5 days, the progress of the infection in the cultures was monitored by measuring the $p24^{gag}$ levels in the supernatants by EIA.

TABLE VI

RABBIT IMMUNIZATION SCHEDULE

Rabbits 238,239 wk 0 : 1° immunization
wk 5 : 2° immunization
wk33 : 3° immunication (only rabbit 238)

241,243 wk 0 : 1° immunization
wk 4 : 2° immunization
wk18 : 3° immunization (only rabbit 241)

IMMUNOGEN	RABBIT	IMMUNIZATION[1] 1°	2°	3°
Recombinant-Made HIV-1 Particles	238	120 ug	190 ug	140 ug
	241	120 ug	100 ug	120 ug
Inactivated HIV-1	239	120 ug	190 ug	
	243	120 ug	100 ug	

[1] Primary immunogens formulated in Complete Freunds Adjuvent and administered intramuscularly; secondary and tertiary immunogens formulated in Incomplete Freunds Adjuvent and administered subcutaneously.

14.2. RESULTS

14.2.1. ANTIBODY TITER DETERMINED BY ELISA

FIG. 14 presents the relative antibody titers in serum

samples collected prior to (Prebleed), as well as 2, 5, and 7 weeks following the 1° immunization as determined by ELISA for rabbits 238 and 239. Rabbit 238 (FIG. 14, graph A) exhibited a hightened immune response to HIV proteins after 5 weeks which was maintained following the 2° immunization (see week 7 data, i.e. 2 weeks post-boost). In contrast, substantial reactivity with HIV proteins was detected 2 weeks folowing 1° immunization in rabbit 239 samples (FIG. 14, graph B), and diminished by 5 weeks. However, a prominent boosting effect in antibody titer was observed in serum sample collected from rabbit 239 at week 7.

One explanation for the poor boost response in rabbit 238 was that the 2° immunization was administered to the animal during the peak immune response (see schedule of bleeds Table VI). In contrast, antibody titer was already declining in rabbit 239 (compare week 2 and week 5 bleeds) when the 2° immunization was administered, thus the boost response was more dramatic.

Presented in FIG. 15 are the results of the ELISA assays conducted on all of the rabbit sera using either immobilized whole disrupted virus (panels 1 and 2) or immobilized purified envelope glycoprotein gp120 (panels 3 and 4). The data presented are the end point titers at different intervals following the primary immunization. The abscissa values represent the weeks post-primary immunizations when serum samples were collected. The arrows indicate the times of the secondary and tertiary immunizations (see Table VI). As shown, both rabbits immunized with the recombinant-made HIV-1 particles (R238 and R241) as well as the rabbits immunized with the psoralen/U.V. inactiviated HIV-1 virions (R239 and R243) generated a profile of antigen reactivity that correlated with the boost schedule. The titer of antibodies in the various rabbit sera reactive with the disrupted whole virus was primarily a reflection of the reactivity with the $p24^{gag}$ protein, which constitutes approximately 90% of the protein

content in both recombinant-made HIV-1 and HIV-1 virions. Reactivity to the whole virus was approxmiately two to three orders of magnitude higher than reactivity to gp120, a disparity directly related to the low levels of the envelope glycoproteins (5-10%) in the particle structures.

14.2.2. NEUTRALIZATION OF HIV-1 INFECTIVITY BY RABBIT ANTISERA

FIG. 16 shows that immunization with the recombinant-made HIV-1 particles generated high titers of neutralizing antibodies to homologous HIV-1 (LAV-1 strain, BRU isolate) (panel A). As with the overall reactivity to viral proteins, the titer of the neutralizing antibodies correlated with the boost schedule. The titers reported correlate with 75% inhibition in $p24^{gag}$ production.

14.2.3. CELLULAR IMMUNE RESPONSE

The cellular immune response elicited by immunization with the recombinant-made particles is summarized in TABLE VII below. These results indicate that lymphocytes isolated from immunized rabbits proliferated in response to specific stimulation with HIV-1 antigens, a response indicative of memory T cell activity necessary in cell-mediated immunity. The stimulation indicies were more significant following the tertiary immunizations in both rabbits immunized with the recombinant-made HIV-1 particles. The very high stimulation index measured for rabbit 243 was correlated with the overall high immune responses measured (FIG. 15, panel B; FIG. 16).

TABLE VII

HIV-SPECIFIC T CELL PROLIFERATIVE RESPONSE IN IMMNIZED RABBITS

IMMUNOGEN	ANIMAL	STIMULATION ANTIGEN	STIMULATION INDEX FIRST BOOST	STIMULATION INDEX SECOND BOOST
Recombinant Particles	238	Inactivated HIV Recomb. Part.	3.0 N.T.	15.0 8.4
	241	Inactivated HIV Recomb. Part.	2.2 N.T.	5.4 N.T.
UV Psoralen Inactivated	239	Inactivated HIV Recomb. Part.	2.6 N.T.	N.B. N.B.
	243	Inactivated HIV Recomb. Part.	95.0 33.0	N.B. N.B.

N.B.: Not Boosted
N.T.: Not Tested

Stimulation index: cpm ^{3}[H]TdR incoroporated into stimulated PBl, divided by cpm ^{3}[H]TdR incorporated into stimulated PBL

14.2.4. WESTERN BLOT ANALYSIS OF ANTIBODY REACTIVITY

FIG. 17 presents the results of an analysis of the reactivity of immunized rabbit sera on viral proteins by Western blot. The results of the Western blot analyses are presented in FIG. 8. The primary immune response in week 5 serum from rabbit 238 was directed against the gag proteins, with only gp41 env protein reactivity (FIG. 17, lane 1). Although no clear boosting effect was detected by ELISA from the week 7 serum sample of rabbit 238, Western blot analysis shows that there was redistribution of antibody response between the various gag proteins, and the emergence of new antibody reactivity with the higher molecular weight HIV

proteins (Fig. 17, lane 2). In contrast, the prominent boosting effect with inactivated virus (FIG. 17, lane 3) was primarily due to enhanced reactivity with the gag proteins as well as the env gp120 protein. Lanes 5, 6 and 7 of FIG. 17 represent reactivity of various concentrations (1:100, 1:1000, and 1:10000, respectively) of pooled human sera from seropositive individuals and serve as control antibody.

15. EXAMPLE: RECOMBINANT-MADE HIV-1 PARTICLES BIND TO AND ARE INTERNALIZED WITHIN CD4+ CELLS

Described herein is an experiment designed to test whether the recombinant-made HIV-1 particles of the invention bind to $CD4^+$ cells and enter by fusion with the plasma membrane as do HIV-1 virions.

15.1. INTERNALIZATION ASSAY

HeLa cells transfected with the gene encoding the CD4 molecule are used in this assay. These cells have been extensively characterized and have been shown to support productive HIV-1 infection. Cells were grown in monolayers on a glass slide in Dulbecco's modified Eagles medium supplemented with 10% fetal calf serum. The cells were washed with PBS/1%FCS and incubated with 50μg/ml recombinant-made HIV-1 particles for 2 hr at 37°C. The cells were then washed extensively and fixed with 3.7% paraformaldehyde solution. The fixed cells were incubated with a 1:100 dilution of a monoclonal antibodies mixture that reacts with the gp120 and $p24^{gag}$ proteins of HIV-1 for 30 min at 22°C. The cells were again washed extensively and incubated with 1:50 dilution of affinity purified goat anti-mouse antibodies conjugated to FITC. After 30 min at 22°C the cells were washed with PBS/1%FCS and mounted in mounting media for viewing. The samples were analyzed by Confocal Laser Scanning Microscopy (CLSM) to reveal intracellular staining.

15.2. RESULTS

The assay results are represented by the CLS micrographs in FIG. 18. FIG. 18A shows a HeLaCD4$^+$ cell exhibiting positive fluorescence following incubation with the recombinant-made HIV-1 particles. FIG. 18B shows the same cell optically sectioned by CLSM, starting from the top of the sample and progressing towards the slide surface in 1μm increments. The micrograph panels show that the fluorescence increases as more of the intracellular environment is revealed, indicating that the recombinant-made HIV-1 particles were internalized following binding.

16. EXAMPLE: IMMUNOGENICITY OF RECOMBINANT-MADE HIV-1 PARTICLES IN NON-HUMAN PRIMATES

The following examines the immunogenicity of recombinant-made HIV-1 particles in a non-human primate species immunized with recombinant-made HIV-1 particles, psoralen/UV-inactivated HIV-1 virions, and recombinant vaccinia virus expressing HIV-1 gag and env antigens. The results demonstrate that the recombinant-made HIV-1 particles elicited both cell-mediated and humoral immune responses, including neutralizing antibodies to HIV-1.

16.1. IMMUNIZATION PROTOCOL

Twelve macaques (Macaca fascicularis) were immunized with recombinant-made HIV-1 particles, psoralen/UV-inactivated HIV-1 virions, or recombinant vaccinia virus expressing HIV-1 gag and env antigens, according to the following schedule:

Group (n=2)	Primary Immunogen/Adjuvant	Secondary Immunogen/Adjuvant	Boosted at week
1.	v-G2E5/none	Particle/IFA	8
2.	v-G2E5/none	rgp160/IFA	8

3.	Particle/IFA	Particle/IFA	4
4.	Particle/DETOX	Particle/DETOX	4
5.	Inact.HIV/IFA	Inact.HIV/IFA	4
6.	Inact.HIV/DETOX	Inact.HIV.DETOX	4

Immunizations with live recombinant vaccinia virus were performed with 1 x 10^7 plaque-forming-units of v-G2E5 Section 7., supra) per animal inoculated by skin-scarification. Each dose of recombinant-made HIV-like particles and psoralen/UV-inactivated HIV virions contained an equivalent of 200 μg of p24 as determined by p24 antigen capture assay (Genetic Systems) and approximately 6 μg of gp120 as determined by immunoblot assay. Recombinant gp160 was purified from BSC-40 cells infected with recombinant vaccinia virus expressing the same antigen and was used at 6 μg per animal per immunization, a dose level similar to that presented by the recombinant-made HIV-1 particles. All particle or subunit immunogens were formulated either in Freund's incomplete adjuvant (Difco) or in DETOX (RIBI Immunobiochem), which containted detoxified monophosphoryl lipid A and bacterial cell wall skeleton. All immunization with recombinant-made particles, inactivated HIV-1 virions, or recombinant gp160 were performed by intramuscular injections.

16.2. RESULTS

Immune responses to HIV-1 generated by these macaques were determined by the following assays: (1) total HIV-1 specific antibodies by whole virion ELISA; (2) HIV-1 envelope specific antibodies by gp120 ELISA; (3) neutralizing antibodies by focal immunoassay; and (4) cell-mediated immunity by lymphoproliferative response to HIV-1 stimulation. The results are summarized in Table VIII.

TABLE VIII

IMMUNE RESPONSES TO HIV-1
GENERATED IMMUNIZED MACAQUES

Group and Immunization		Animal	Antibody Response[a] HIV	gp120	Neut	S.I.[b]
1	v-G2E5/	89133	>25600	>1600	>400	2.1
	Particle	89149	12800	>1600	>400	115.4
2	v-G2E5/	89147	1600	400	25	74.3
	gp160	89138	0	800	100	69.7
3	Particle	88079	>51200	>800	25	3.9
	(IFA)	89068	6400	>1600	25	50.0
4	Particle	88116	800	1600	25	2.8
	(DETOX)	89072	0	<25	0	4.6
5	Iact.HIV	89150	12800	800	<25	4.7
	(IFA)	89086	3200	<25	0	43.3
6	Iact.HIV	89151	1600	1600	25	5.7
	(DETOX)	89156	50	800	0	3.9
Control Monkey			0	0	0	0.9

[a]Antibody response was determined at 4 weeks after the secondary immunization, i.e. week 12 for animals in Groups 1 and 2 and week 8 for animals in Groups 3 to 6.

[b]Lymphoproliferative response was determined at 4 weeks after the primary immunization. Stimulation index (S.I.) was determined by dividing the c.p.m. ^{3}H-thymidine incorporated into HIV-1-stimulated cells by the c.p.m. incorporated into non-stimulated cells.

The results presented in Table VI indicate that: (1) recombinant-made HIV-1 particles are immunogenic and can elicit an HIV-specific immune response, particularly when used in animals previously primed with recombinant vaccinia virus expressing both env and gag antigens; (2) boosting previously primed animals with recombinant-made HIV-1 particles was more effective at eliciting an immune response than boosting with equivalent amounts of soluble gp160, most likly due to the effective presentation of envelope antigens by the recombinant-made HIV-1 particles; and (3) when used as the sole immunogen for primary and secondary immunizations, recombinant-made HIV-1 particles elicit HIV-specific antibody and T-cell immune responses in the majority of immunized macaques at levels similar to those immunized with equivalent does of inactivated HIV-1 virions.

17. DEPOSIT OF MICROORGANISMS

The following recombinant vaccine viruses have been deposited with the American Type Culture Collection, Rockville, MD, and have been assigned the listed accession numbers:

Recombinant Virus	Accession Number
v-env5	VR 2113
v-gag2	VR 2265
v-G2E5	

The following recombinant plasmids have been deposited with the NRRL, Peoria, Illinois and have been assigned the listed accession numbers.

Plasmid	Host	Accession No
CMHiEnv5(1104-b1)	E. coli DH5a	
CmHIVdelKpnAvr (Gag2TRE) (1160-a1)	E. coli DH5a	

The present invention is not to be limited in scope by the viruses deposited since the deposited embodiments are intended as single illustrations of one aspect of the invention and any which are functionally equivalent are within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

It is also to be understood that all base pair and amino acid residue numbers and sizes given for nucleotides and peptides are approximate and are used for purposes of description.

Nucleotide positions given for HIV-1 genes are based on the genomic sequence of the LAV-BRU isolate, Genebank accession no. K02013 (Wain-Hobson et al., 1985, Cell 40:9317).

WHAT IS CLAIMED:

1. A nonreplicating recombinant-made HIV particle comprising assembled core and envelope proteins incorporating a plurality of immunogenic epitopes of native Human Immunodeficiency Virus.

2. The nonreplicating recombinant-made HIV particle of Claim 1 in which the core proteins comprise Human Immunodeficiency Virus Type 1 core proteins.

3. The nonreplicating recombinant-made HIV particle of Claim 2 in which the Human Immunodeficiency Virus Type 1 core proteins comprise p24.

4. The nonreplicating recombinant-made HIV particle of Claim 2 in which the Human Immunodeficiency Virus Type 1 core proteins comprise p24 and p17.

5. The nonreplicating recombinant-made HIV particle of Claim 1 in which the envelope proteins comprise Human Immunodeficiency Virus Type 1 envelope proteins.

6. The nonreplicating recombinant-made HIV particle of Claim 5 in which the HIV-1 envelope proteins comprise gp41.

7. The nonreplicating recombinant-made HIV particle of Claim 5 in which the Human Immunodeficiency Virus Type 1 envelope proteins comprise gp41 and gp120.

8. The nonreplicating recombinant-made HIV particle of Claim 5 in which the HIV-1 envelope proteins comprise gp160.

9. The nonreplicating recombinant-made HIV particle of Claim 8 in which the gp160 is not cleaved to yield gp120 and gp41.

10. The nonreplicating recombinant-made HIV particle of Claim 8 in which the gp160 is truncated.

11. The nonreplicating recombinant-made HIV particle of Claim 9 in which the gp160 is truncated.

12. The nonreplicating recombinant-made HIV particle of Claim 1 in which the core proteins comprise HIV-2 core proteins.

13. The nonreplicating recombinant-made HIV particle of Claim 1 in which the envelope proteins comprise HIV-2 envelope proteins.

14. The nonreplicating recombinant-made HIV particle of Claim 1 in which the envelope proteins comprise both HIV-1 and HIV-2 envelope proteins.

15. A nonreplicating recombinant-made HIV-1 particle comprising assembled HIV-1 core and envelopes proteins incorporating a plurality of immunogenic epitopes of native HIV-1.

16. A nonreplicating recombinant-made HIV-2 particle comprising assembled HIV-2 core and envelope proteins incorporating a plurality of immunogenic epitopes of native HIV-2.

17. A nonreplicating recombinant-made HIV particle comprising assembled HIV core and envelope proteins incorporating a plurality of immunogenic epitopes of both native HIV-1 and native HIV-2.

18. A method for inhibiting the progression of Acquired Immunodeficiency Syndrome in an individual infected with Human Immunodeficiency Virus comprising administering nonreplicating recombinant-made HIV particles to the individual in an amount effective at inhibiting Human Immunodeficiency Virus infection.

19. A method for inhibiting the progression of lymphadenopathy in an individual infected with Human Immunodeficiency Virus comprising administering nonreplicating recombinant-made HIV particles to the individual in an amount effective at inhibiting Human Immunodeficiency Virus infection.

20. A method for inhibiting the progression of AIDS-Related Complex in an individual infected with Human Immunodeficiency Virus comprising administering nonreplicating recombinant-made HIV particles to the individual in an amount effective at inhibiting Human Immunodeficiency Virus infection.

21. A method for reducing the infectivity of $CD4^+$ lymphocytes by Human Immunodeficiency Virus comprising treating the lymphocytes with nonreplicating recombinant-made HIV particles in an amount effective at inhibiting Human Immunodeficiency Virus infection.

22. A nonreplicating recombinant-made retroviral particle comprising assembled retroviral core and envelope proteins incorporating a plurality of immunogenic epitopes of native human retrovirus.

23. The nonreplicating recombinant-made retroviral particle of Claim 22 in which the retroviral core proteins comprise HTLV-I core proteins.

24. The nonreplicating recombinant-made retroviral particle of Claim 22 in which the retroviral core proteins comprise HTLV-II core proteins.

25. The nonreplicating recombinant-made retroviral particle of Claim 22 in which the retroviral envelope proteins comprise HTLV-I envelope proteins.

26. The nonreplicating recombinant-made retroviral particle of Claim 22 in which the retroviral envelope proteins comprise HTLV-II envelope proteins.

27. The nonreplicating recombinant-made retroviral particle of Claim 22 in which the retroviral envelope proteins comprises both HTLV-I and HTLV-II envelope proteins.

28. A nonreplicating recombinant-made HTLV-I particle comprising assembled HTLV-I core and envelope proteins incorporating a plurality of immunogenic epitopes of native HTLV-I.

29. A nonreplicating recombinant-made HTLV-II particle comprising assembled HTLV-II core and envelope proteins incorporating a plurality of immunogenic epitopes of native HTLV-II.

30. A nonreplicating recombinant-made HTLV particle comprising assembled HTLV core and envelope proteins incorporating a plurality of immunogenic epitopes of both native HTLV-I and HTLV-II.

31. A method for inhibiting the progression of Adult T Cell Leukemia in an individual infected with HTLV-I comprising administering nonreplicating recombinant-made HTLV-I particles to the individual in an amount effective at

inhibiting HTLV-I infection.

32. A method for inhibiting the progression of HTLV-I-associated lymphoma in an individual infected with HTLV-I comprising administering nonreplicating recombinant-made HTLV-I particles to the individual in an amount effective at inhibiting HTLV-I infection.

33. A method for inhibiting the progression of HTLV-II-associated leukemia in an individual infected with HTLV-II comprising administering nonreplicating recombinant-made HTLV-II particles to the individual in an amount effective at inhibiting HTLV-II infection.

34. A method for reducing the infectivity of $CD4^+$ lymphocytes by a human retrovirus comprising treating the lymphocytes with nonreplicating recombinant-made retroviral particles capable of binding to the CD4 cell surface receptor in an amount effective at inhibiting human retrovirus infection.

35. A method for generating nonreplicating-made retroviral particles comprising:

(a) introducing nucleotide sequences encoding retroviral core, protease, and envelope proteins into a mammalian host cell;

(b) coexpressing mature retroviral core and envelope proteins within the mammalian host cell;

(c) culturing the mammalian host cell; and

(d) recovering the nonreplicating recombinant-made retroviral particles from the culture medium.

36. The method according to Claim 35 in which the nucleotide sequences encoding retroviral core, protease, and envelope proteins are introduced into the mammalian cell by infection with at least one live viral vector.

37. The method according to Claim 36 in which the live viral vectors comprise recombinant vaccinia virus.

38. The method according to Claim 36 in which the live viral vector comprises a recombinant retrovirus.

39. The method according to Claim 35 in which the nucleotide sequences encoding retroviral core, protease, and envelope proteins are introduced into the mammalian cell by transfection with at least one DNA vector.

40. A method for generating nonreplicating recombinant-made HIV particles comprising:

(a) coinfecting a mammalian host cell with (i) at least one recombinant vaccinia virus carrying an HIV env gene and (ii) at least one recombinant vaccinia virus carrying HIV gag and protease genes;

(b) coexpressing mature HIV env and gag encoded gene products within the infected mammalian host cell;

(c) culturing the infected mammalian host cell; and

(d) recovering the nonreplicating recombinant-made HIV particles from the culture medium.

41. The method according to Claim 60 further comprising infecting the mammalian host cell with at least one recombinant vaccinia virus carrying at least one HIV gene selected from the group tat, rev, vif, vpr and vpu.

42. The method according to Claims 40 or 41 in which the HIV env gene is a HIV-1 env gene.

43. The method according to Claims 40 or 41 in which the HIV env gene is a HIV-2 env gene.

44. The method according to Claims 40 or 41 in which the HIV env gene is a HIV-1 gag gene.

45. The method according to Claims 40 or 41 in which the HIV env gene is a HIV-2 gag gene.

46. The method according to Claims 40 or 41 in which the HIV protease gene is a HIV-1 protease gene.

47. The method according to Claims 40 or 41 in which the HIV protease gene is a HIV-2 protease gene.

48. A method for generating nonreplicating recombinant-made HIV particles comprising:

(a) infecting a mammalian host cell with a recombinant vaccinia virus carrying HIV env, gag and protease genes;

(b) coexpressing mature HIV env and gag encoded gene products within the infected mammalian host cell;

(c) culturing the infected mammalian host cell; and

(d) recovering the nonreplicating recombinant-made HIV particles from the culture medium.

49. The method according to Claim 48 further comprising infecting the mammalian host cell with at least one recombinant vaccinia virus carrying at least one HIV gene selected from the group tat, rev, vif, vpr, and vpu.

50. The method according to Claims 48 or 49 in which the HIV env gene is an HIV-1 env gene.

51. The method according to Claims 48 or 49 in which the HIV env gene is an HIV-2 env gene.

52. The method according to Claims 48 or 49 in which the HIV gag gene is an HIV-1 gag gene.

53. The method according to Claims 48 or 49 in which the HIV gag gene is an HIV-2 gag gene.

54. The method according to Claims 48 or 49 in which the HIV protease gene is an HIV-1 protease gene.

55. The method according to Claims 48 or 49 in which the HIV protease gene is an HIV-2 protease gene.

56. A method for generating nonreplicating recombinant-made HIV particles comprising:

(a) transfecting a mammalian host cell with a recombinant plasmid carrying HIV env, gag, and protease genes;

(b) coexpressing mature HIV env and gag encoded gene products within the transfected

mammalian host cell;

(c) culturing the transfected mammalian host host cell; and

(d) recovering the nonreplicating recombinant-made HIV particles from the culture medium.

57. The method according to Claim 56 in which the recombinant plasmid also carries at least one other HIV gene selected from the group tat, rev, vif, vpr and vpu.

58. The method according to Claim 56 further comprising transfecting the mammalian host cell with at least one recombinant plasmid carrying at least one HIV gene selected from the group tat, rev, vif, vpr and vpu.

59. The method according to Claims 56, 57 or 58 in which the HIV env gene is an HIV-1 env gene.

60. The method according to Claims 56, 57 or 58 in which the HIV env gene is an HIV-2 env gene.

61. The method according to Claims 56, 57 or 58 in which the HIV gag gene is a HIV-1 gag gene.

62. The method according to Claims 56, 57 or 58 in which the HIV gag gene is an HIV-2 gag gene.

63. The method according to Claims 56, 57 or 58 in which the HIV protease gene is an HIV-1 protease gene.

64. The method according to Claim 56, 57 or 58 in which the HIV protease gene is an HIV-2 protease gene.

65. A method for generating nonreplicating

recombinant-made HIV particles comprising:

(a) transfecting a mammalian host cell with (i) a recombinant plasmid carrying at least and HIV env gene and (ii) a recombinant plasmid carrying at least HIV gag and protease genes;

(b) coexpressing mature HIV env and gag encoded gene products within the transfected mammalian host cells;

(c) culturing the transfected mammalian host cell; and

(d) recovering the nonreplicating recombinant-made HIV particles from the culture medium.

66. The method according to Claim 65 further comprising transfecting the mammalian host cell with at least one recombinant plasmid carrying at least one HIV gene selected from the group tat, rev, vif, vpr and vpu.

67. The method according to Claims 65 or 66 in which the HIV env gene is an HIV-1 env gene.

68. The method according to Claims 65 or 66 in which the HIV env gene is an HIV-2 env gene.

69. The method according to Claims 65 or 66 in which the HIV gag gene is an HIV-1 gag gene.

70. The method according to Claims 65 or 66 in which the HIV gag gene is an HIV-2 gag gene.

71. The method according to Claims 65 or 66 in which the HIV protease gene is an HIV-1 protease gene.

72. The method according to Claims 65 or 66 in which the HIV protease gene is an HIV-2 protease gene.

73. The method according to Claims 40, 48, 56 or 65 in which the HIV env gene encodes an uncleaved gp160 protein.

74. The method according to Claims 40, 48, 56 or 65 in which the HIV env gene encodes a truncated gp160 protein.

75. A method for generating nonreplicating recombinant-made HIV-1 particles comprising:

(a) coinfecting a BSC-40 host cell with recombinant vaccinia viruses v-env5 and v-gag2 as deposited with the ATCC:

(b) culturing the infected BSC-40 cell; and

(c) recovering the nonreplicating recombinant-made HIV-1 particles from the culture medium.

76. A method for generating nonreplicating recombinant-made HIV particles comprising:

(a) coexpressing HIV env encoded and HIV gag encoded structural proteins in mammalian cells; and

(b) recovering the nonreplicating recombinant-made HIV particles from the culture medium.

77. A method for generating nonreplicating recombinant-made HIV-1 particles comprising:

(a) infecting a BSC-40 host cell with recombinant vaccinia virus v-G2E5 as deposited with the ATCC;

(b) culturing the infected BSC-40 cell; and

(c) recovering the nonreplicating recombinant-made HIV-1 particles form the culture medium.

78. A method for generating nonreplicating recombinant-made HIV-1 particles comprising:

(a) coinfecting a BSC-40 host cell with (i) recombinant vaccinia virus v-ED2 and (ii) recombinant vaccinia virus v-gag2;

(b) culturing the infected BSC-40 cell; and

(c) recovering the nonreplicating recombinant-made HIV-1 particles form the culture medium.

79. A method for generating nonreplicating recombinant-made HIV-1 particles comprising:

(a) coinfecting a BSC-40 host cell with (i) recombinant vaccinia virus v-ENV5DCT and (ii) recombinant vaccinia virus v-gag2;

(b) culturing the infected BSC-40 cell; and

(c) recovering the nonreplicating recombinant-

made HIV-1 particles form the culture medium.

80. A method for generating nonreplicating recombinant-made HIV-1 particles comprising:

(a) coinfecting a BSC-40 host cell with (i) recombinant vaccinia virus v-160NC and (ii) recombinant vaccinia virus v-gag2;

(b) culturing the infected BSC-40 cell; and

(c) recovering the nonreplicating recombinant-made HIV-1 particles form the culture medium.

81. A method for generating nonreplicating recombinant-made HIV-1 particles comprising:

(a) coinfecting a BSC-40 host cell with (i) recombinant vaccinia virus v-11K160NC and (ii) recombinant vaccinia virus v-gag2;

(b) culturing the infected BSC-40 cell; and

(c) recovering the nonreplicating recombinant-made HIV-1 particles form the culture medium.

82. A method for generating nonreplicating recombinant-made HIV-1 particles comprising:

(a) cotransfecting a mammalian cell with recombinant plasmids CmHIVdelKpnAvr(Gag2TRE) (1160-a1) and CmHiEnv5 (1104-b1) as deposited with the NRRL;

(b) coexpressing mature HIV env and gag encoded gene products within the transfected CHO cells;

(c) culturing the transfected CHO cells; and

(d) recovering the nonreplicating recombinant-made HIV-1 particles from the culture medium.

83. A method for generating nonreplicating recombinant-made HIV-1 particles comprising:

(a) transfecting a mammalian cell with recombinant plasmids CmHIVdelKpnAvr(Gag2TRE) (1160-a1) as deposited with the NRRL;

(b) coexpressing mature HIV env and gag encoded gene products within the transfected CHO cells;

(c) culturing the transfected CHO cells; and

(d) recovering the nonreplicating recombinant-made HIV-1 particles from the culture medium.

84. A method for generating nonreplicating recombinant-made HIV-1 particles comprising:

(a) cotransfecting a mammalian cell with recombinant plasmids CmHiGag2Rre(1158-a1) and CmHIVdelXmn(1133-a1) as deposited with the NRRL;

(b) coexpressing mature HIV env and gag encoded

gene products within the transfected CHO cells;

(c) culturing the transfected CHO cells; and

(d) recovering the nonreplicating recombinant-made HIV-1 particles from the culture medium.

85. A method for generating nonreplicating recombinant-made HIV-1 particles comprising:

(a) cotransfecting a mammalian cell with recombinant plasmids CmHIVdelXmn(1133-a1) and CmvGag2Rre(1159-a1) as deposited with the NRRL;

(b) coexpressing mature HIV env and gag encoded gene products within the transfected CHO cells;

(c) culturing the transfected CHO cells; and

(d) recovering the nonreplicating recombinant-made HIV-1 particles from the culture medium.

86. A method for generating nonreplicating recombinant-made HIV-1 particles comprising:

(a) cotransfecting a mammalian cell with recombinant plasmids CmHiGag2Rre, CmHiEnv5, CmHiTat and CmHiRev as deposited with the NRRL;

(b) coexpressing mature HIV env and gag encoded

gene products within the transfected CHO cells;

(c) culturing the transfected CHO cells; and

(d) recovering the nonreplicating recombinant-made HIV-1 particles from the culture medium.

FIG. 1

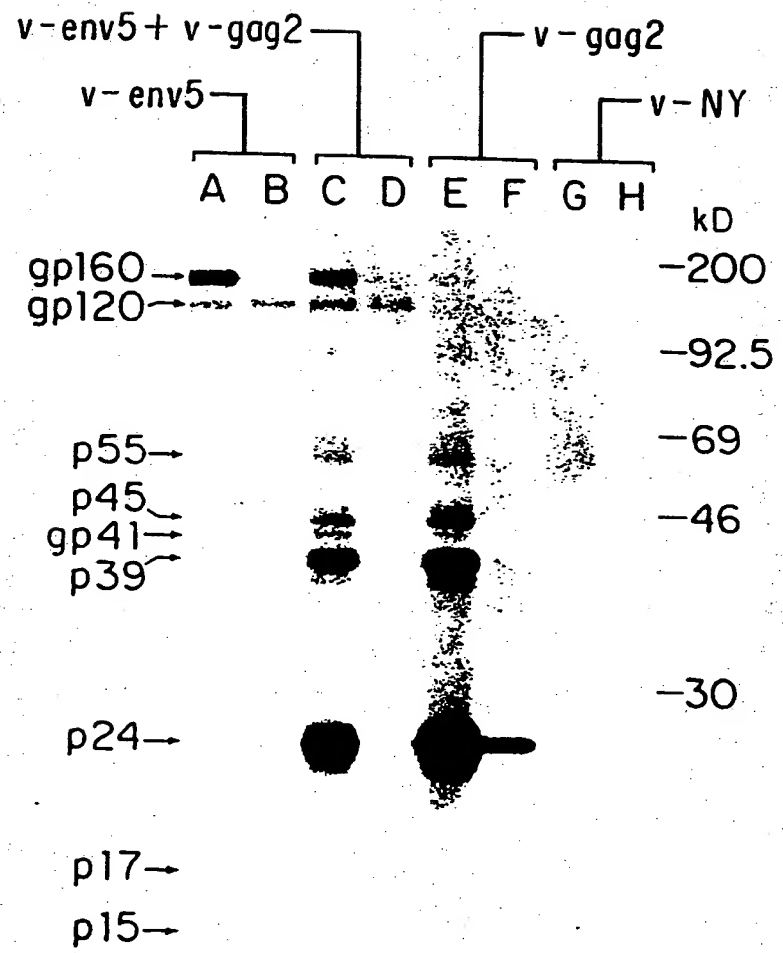
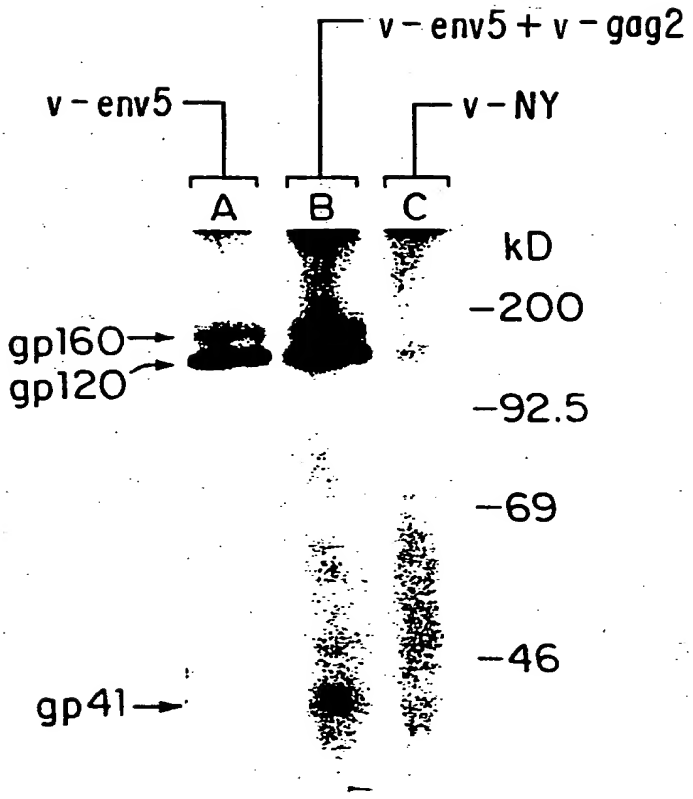

v-env5 + v-gag2
v-env5
v-gag2
v-NY
A B C D E F G H
kD
gp160
gp120
p55
p45
gp41
p39
p24
p17
p15
-200
-92.5
-69
-46
-30

FIG. 2



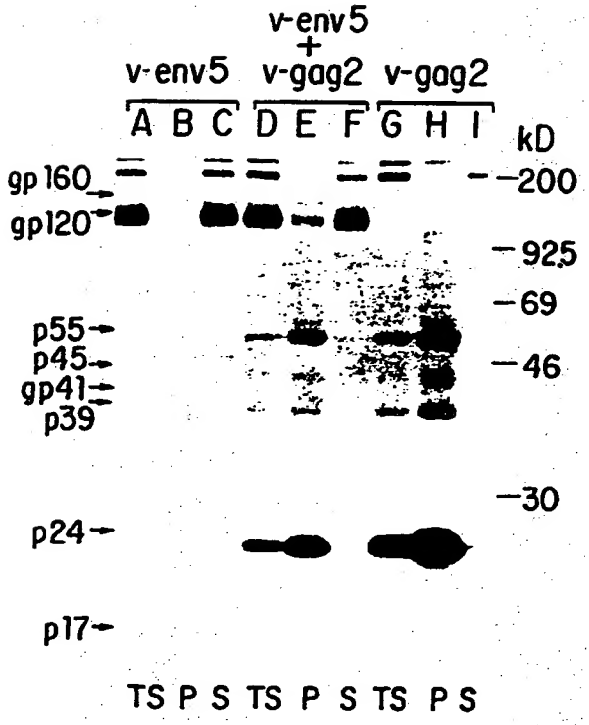

v-env5
+
v-gag2
v-env5
v-gag2
A B C D E F G H I
kD
gp160
gp120
p55
p45
gp41
p39
p24
p17
200
925
69
46
30
TS P S TS P S TS P S

FIG. 3A

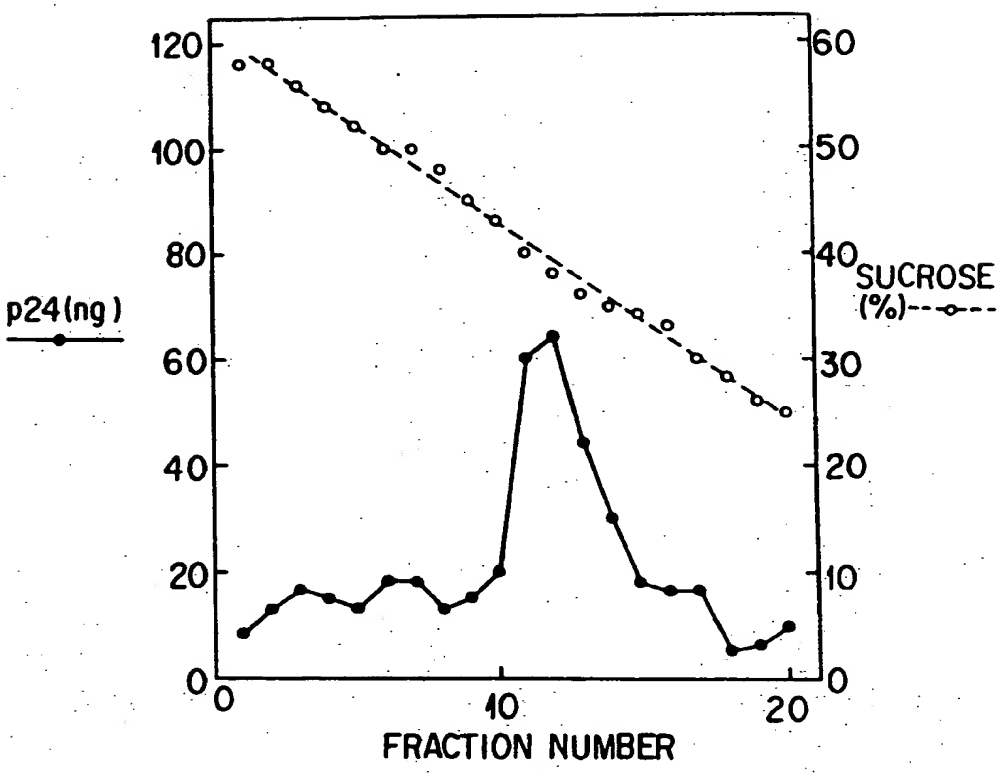

120
100
80
60
40
20
0
60
50
40
30
20
10
0
0
10
20
FRACTION NUMBER
p24(ng)
SUCROSE
(%)

FIG. 3B

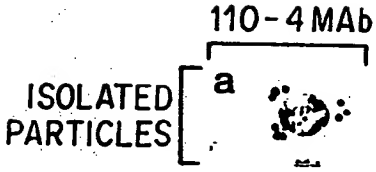


FIG. 4a

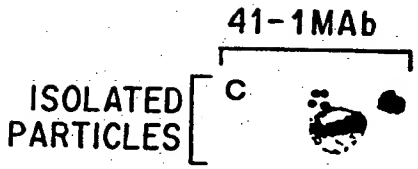


FIG. 4c

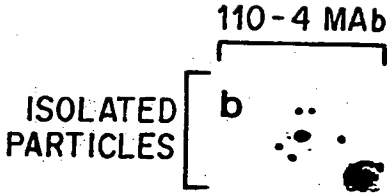


FIG. 4b

FIG. 4d

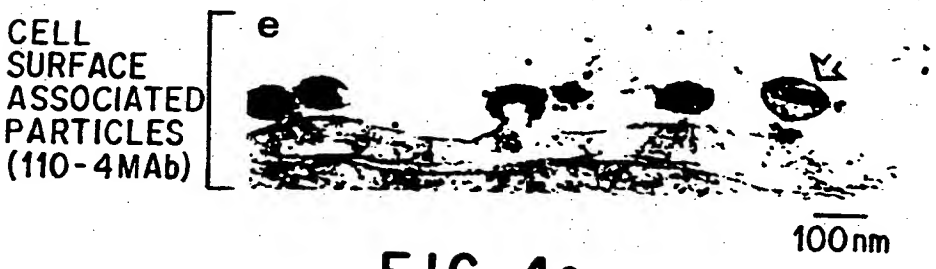


FIG. 4e

FIG. 5A

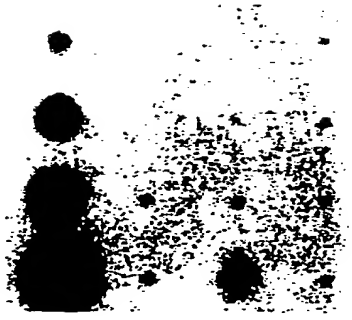


FIG. 5B

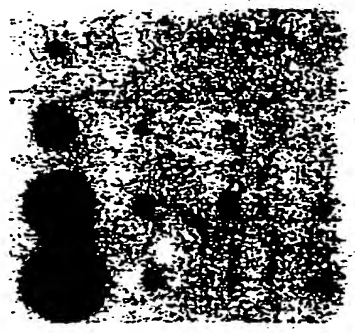
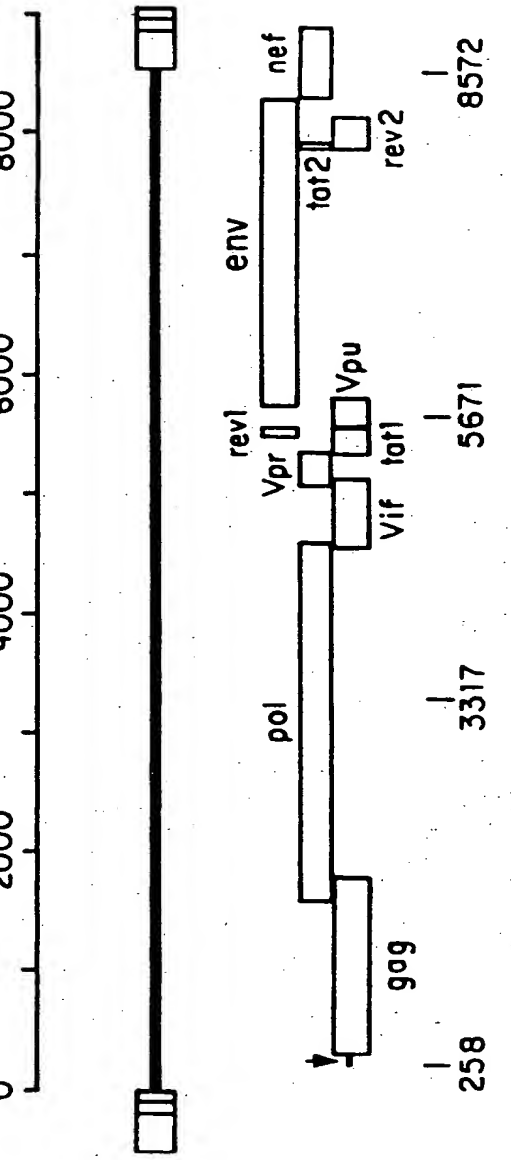


FIG. 5C



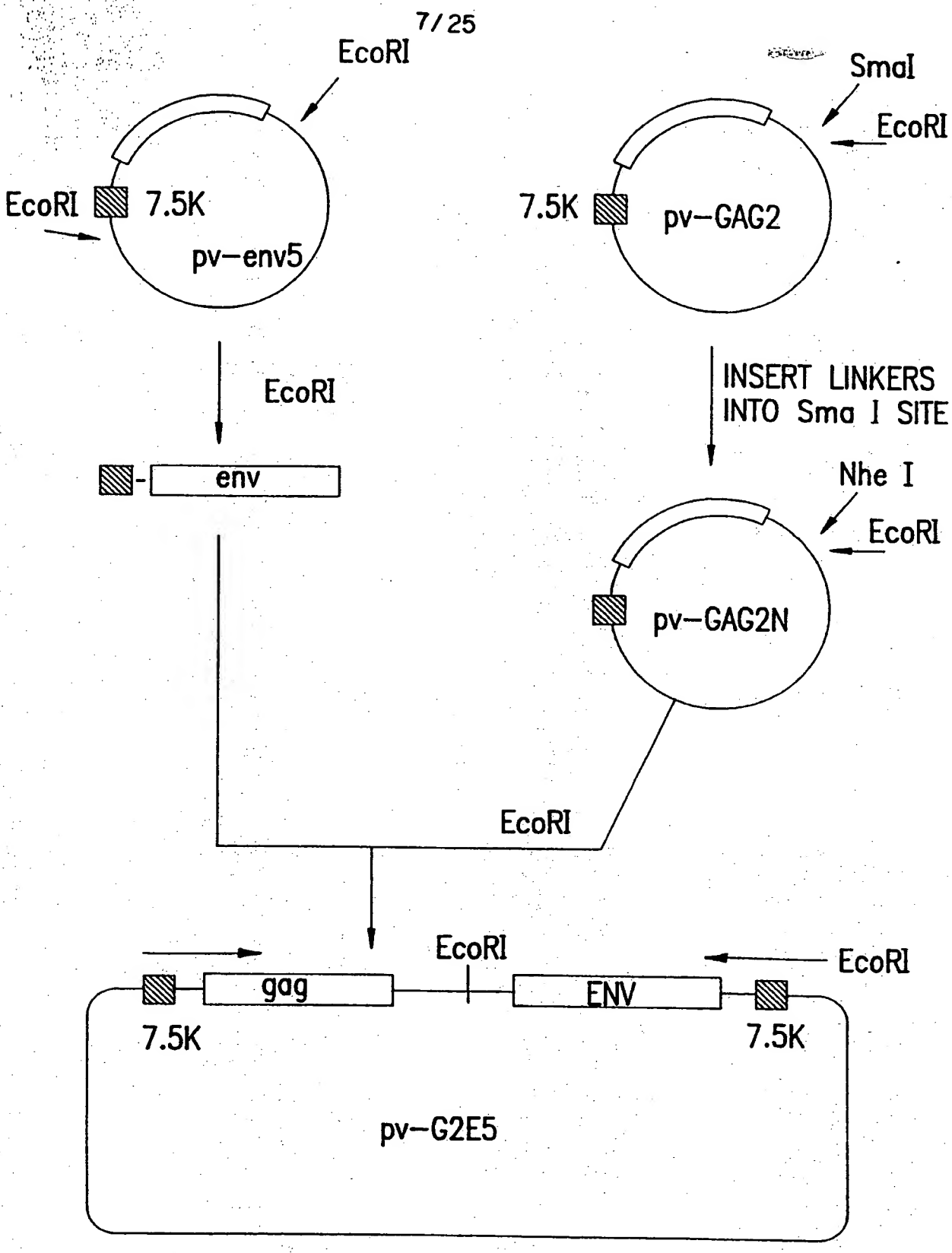

EcoRI
EcoRI
7.5K
pv-env5
SmaI
EcoRI
7.5K
pv-GAG2
EcoRI
INSERT LINKERS
INTO Sma I SITE
env
Nhe I
EcoRI
pv-GAG2N
EcoRI
EcoRI
EcoRI
gag
ENV
7.5K
7.5K
pv-G2E5

FIG. 6

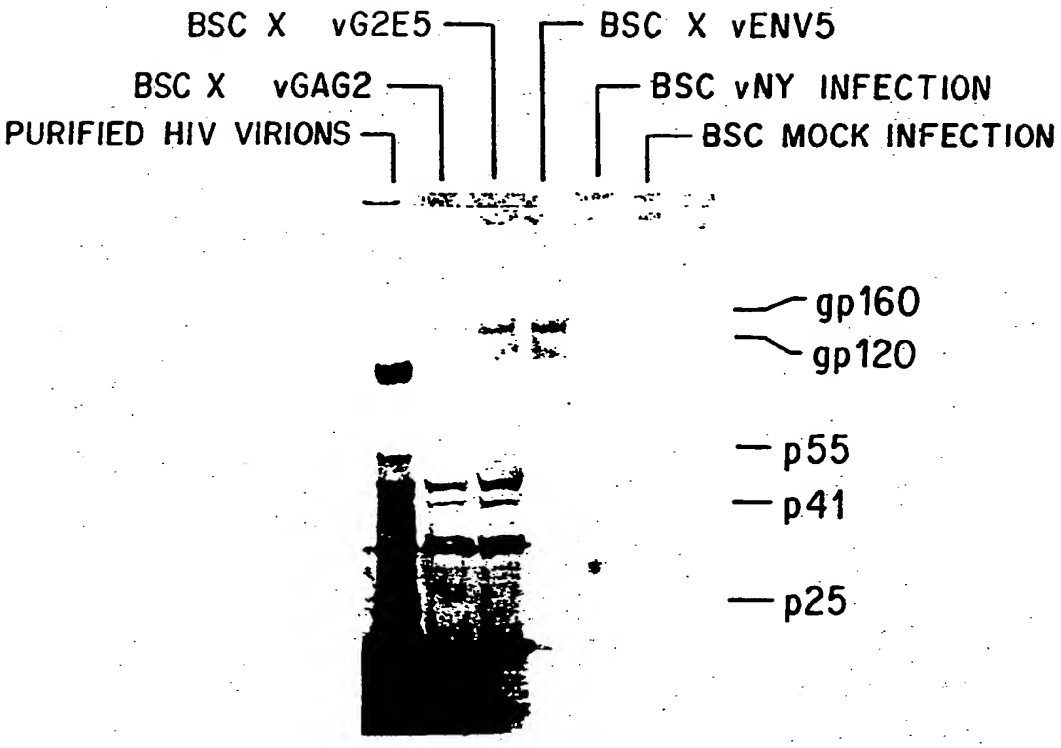


FIG. 7

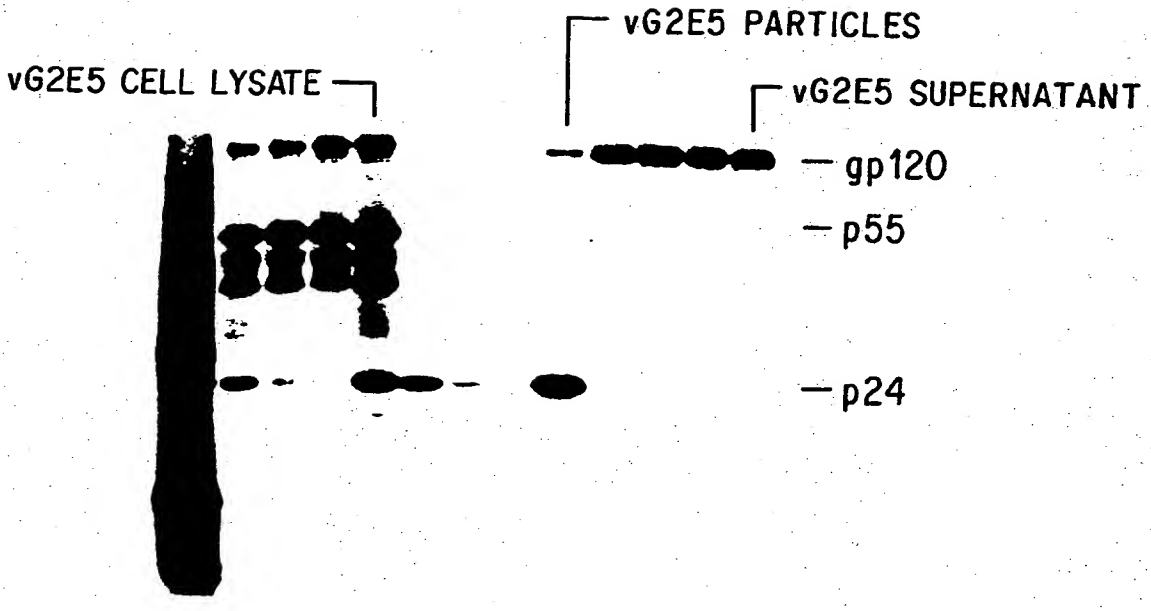

vG2E5 PARTICLES
vG2E5 CELL LYSATE
vG2E5 SUPERNATANT
gp120
p55
p24

FIG. 8

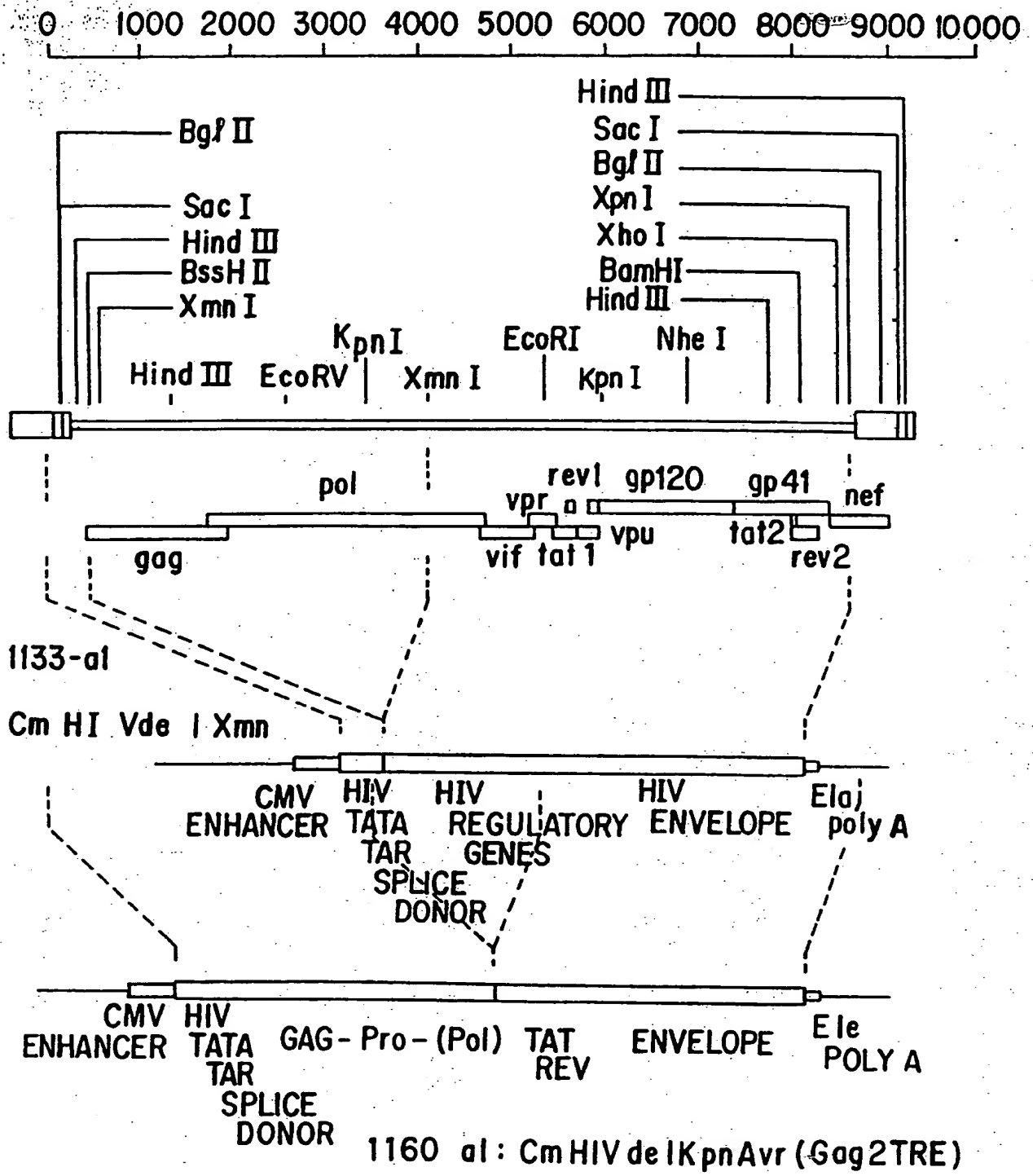


FIG. 9A

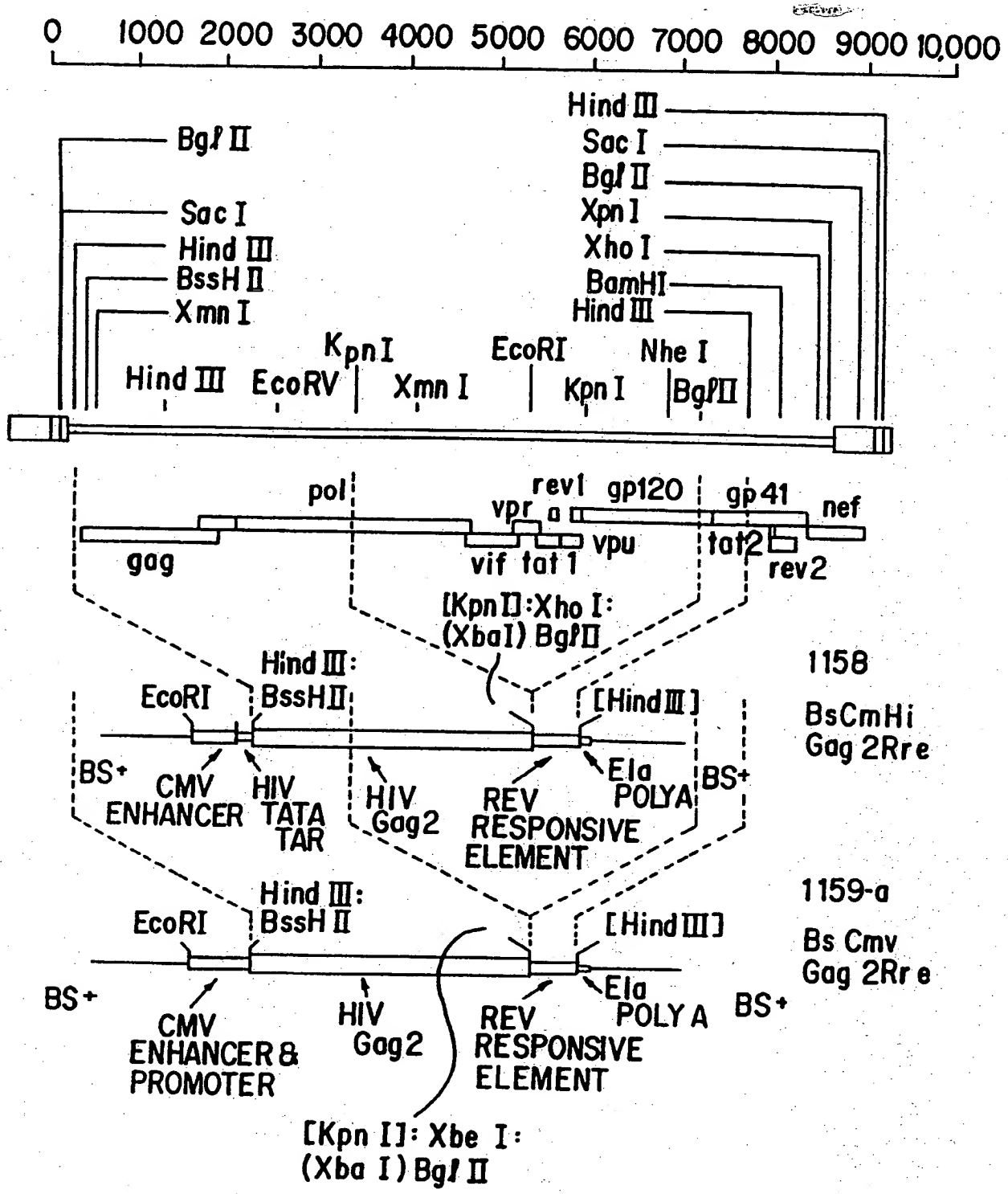


FIG. 9B

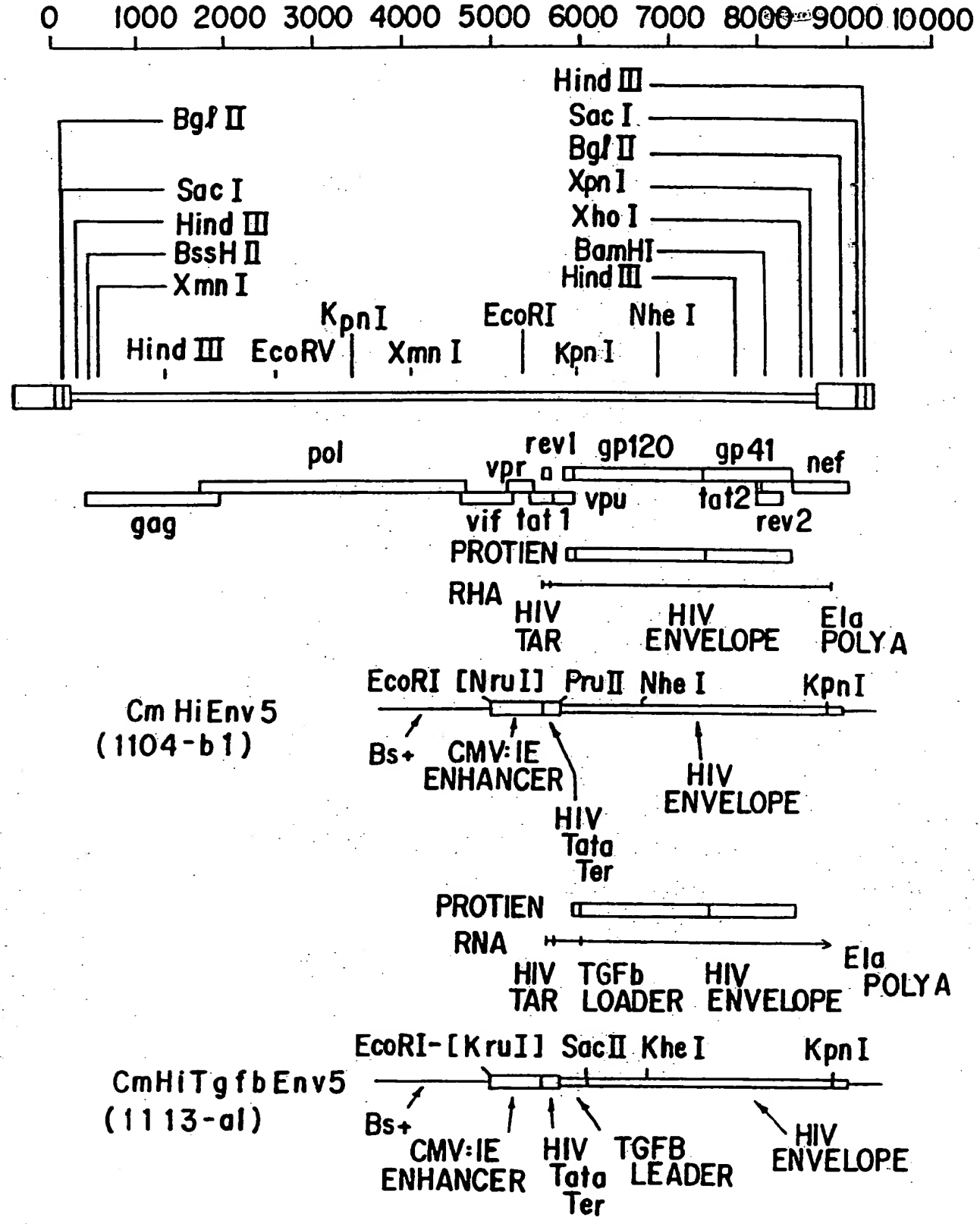


FIG. 9C

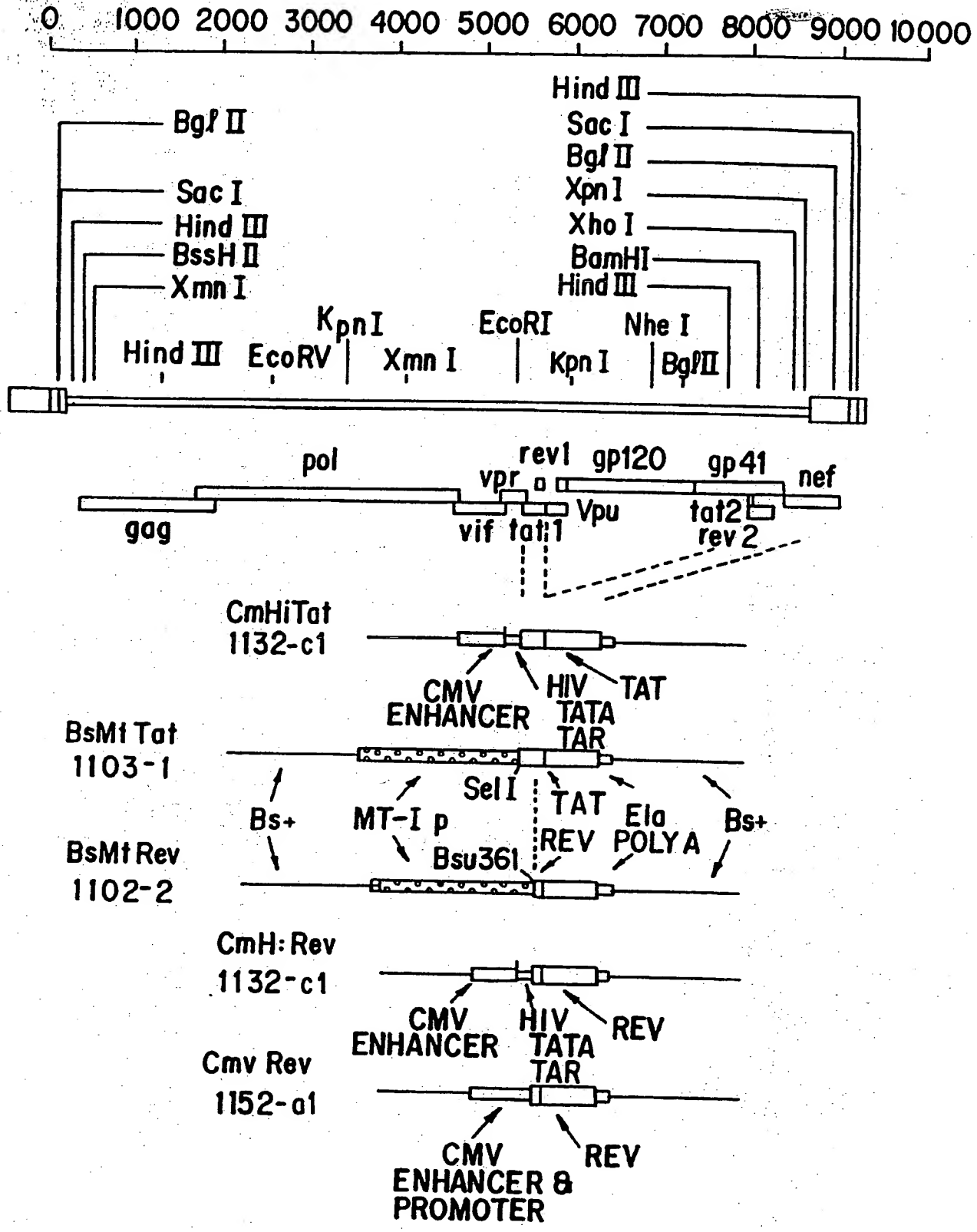

0 1000 2000 3000 4000 5000 6000 7000 8000 9000 10000
Bgl II
Sac I
Hind III
BssH II
Xmn I
Hind III
Sac I
Bgl II
Xpn I
Xho I
BamHI
Hind III
Hind III
EcoRV
Kpn I
Xmn I
EcoRI
Kpn I
Nhe I
Bgl II
pol
gag
vpr
vif
rev1
tat1
Vpu
gp120
gp41
nef
tat2
rev 2
CmHiTat
1132-c1
CMV
ENHANCER
HIV
TATA
TAT
TAR
BsMt Tat
1103-1
Sel I
Bs+
MT-I p
TAT
REV
Ela
POLY A
Bs+
BsMt Rev
1102-2
Bsu361
CmH: Rev
1132-c1
CMV
ENHANCER
HIV
TATA
TAR
REV
Cmv Rev
1152-a1
CMV
ENHANCER &
PROMOTER
REV

FIG. 9D

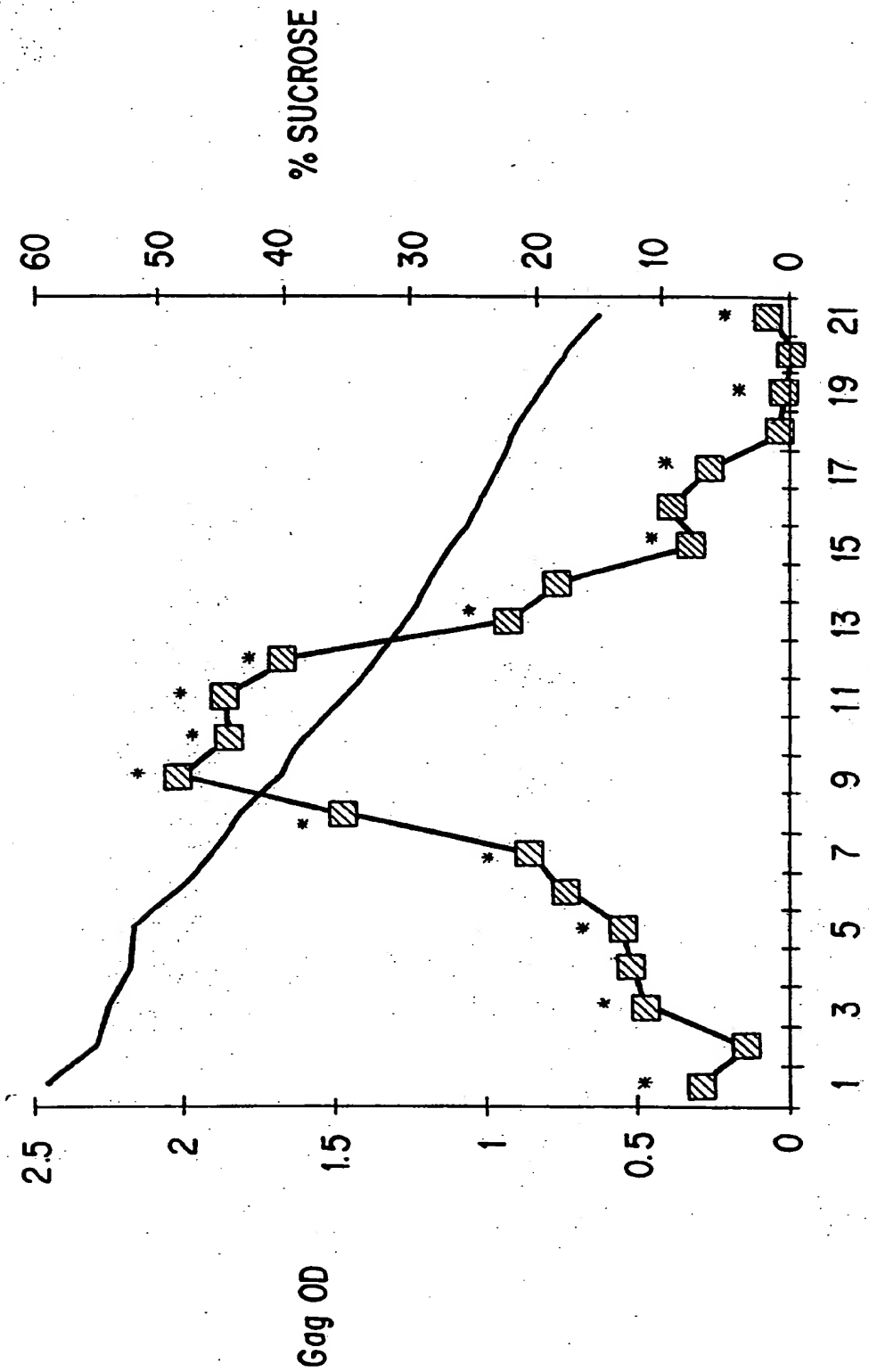

% SUCROSE
60
50
40
30
20
10
0
Gag OD
2.5
2
1.5
1
0.5
0
1
3
5
7
9
11
13
15
17
19
21
BOTTOM
FRACTION #
TOP

FIG. 10A

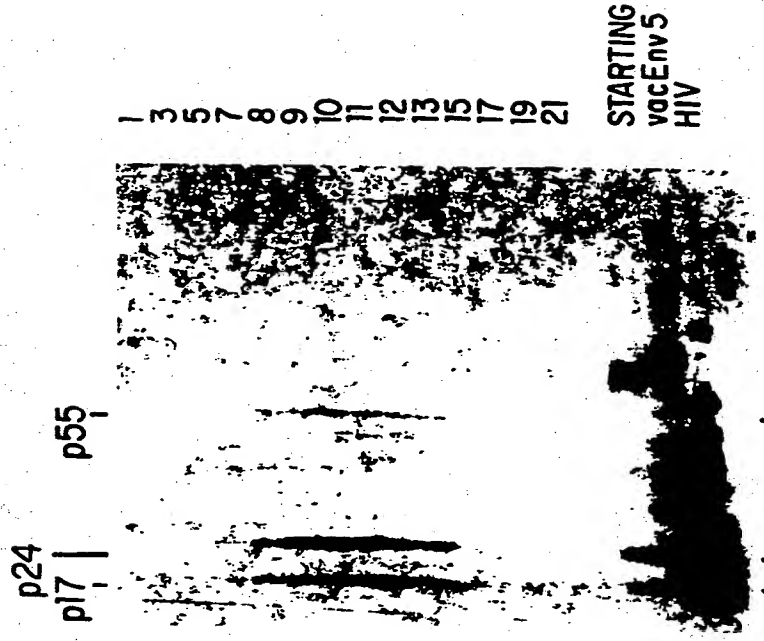

1 3 5 7 8 9 10 11 12 13 15 17 19 21
STARTING
vacEnv5
HIV
p55
p24
p17
p55
p17
p24

FIG. 10B

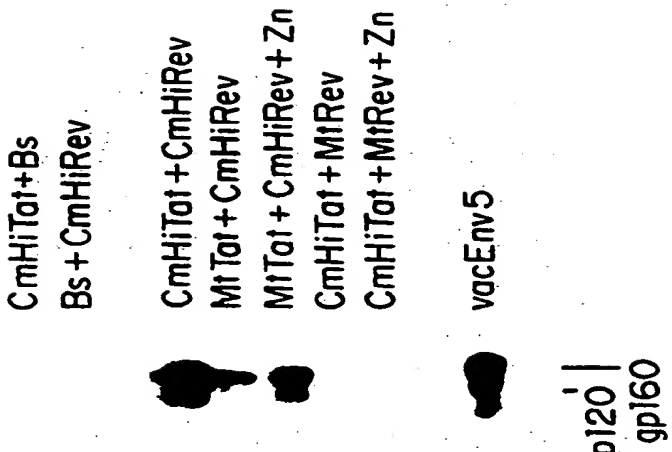


FIG. 11

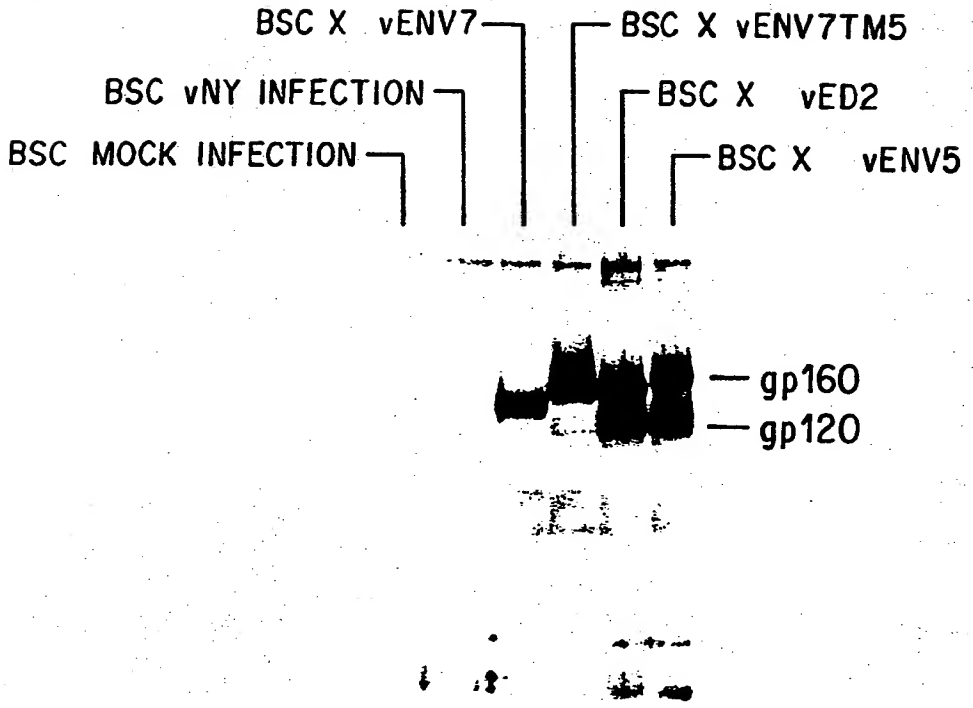


FIG. 12

RECOMB. PART.

FIG. 13A

HIV - 1

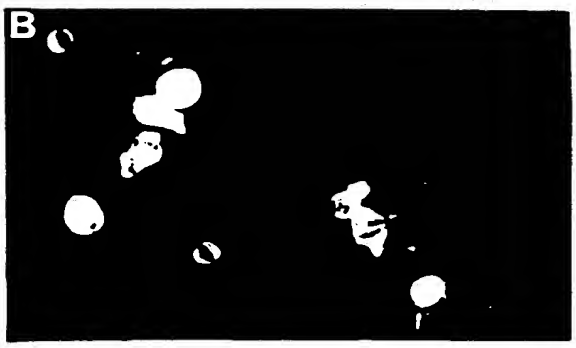


FIG. 13B

HIV - 1

FIG. 13C

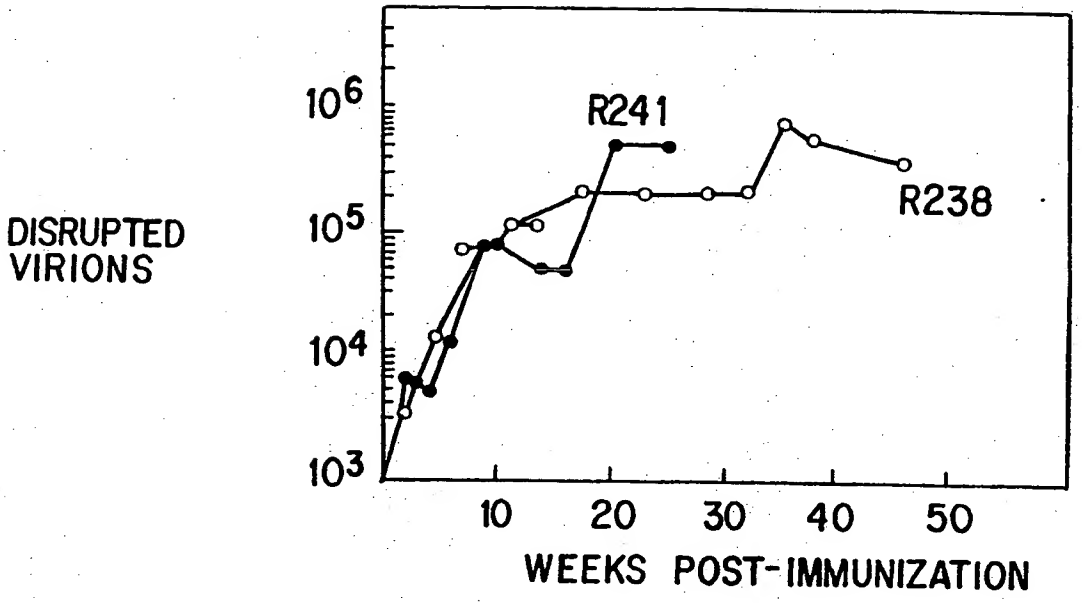


FIG. 15A

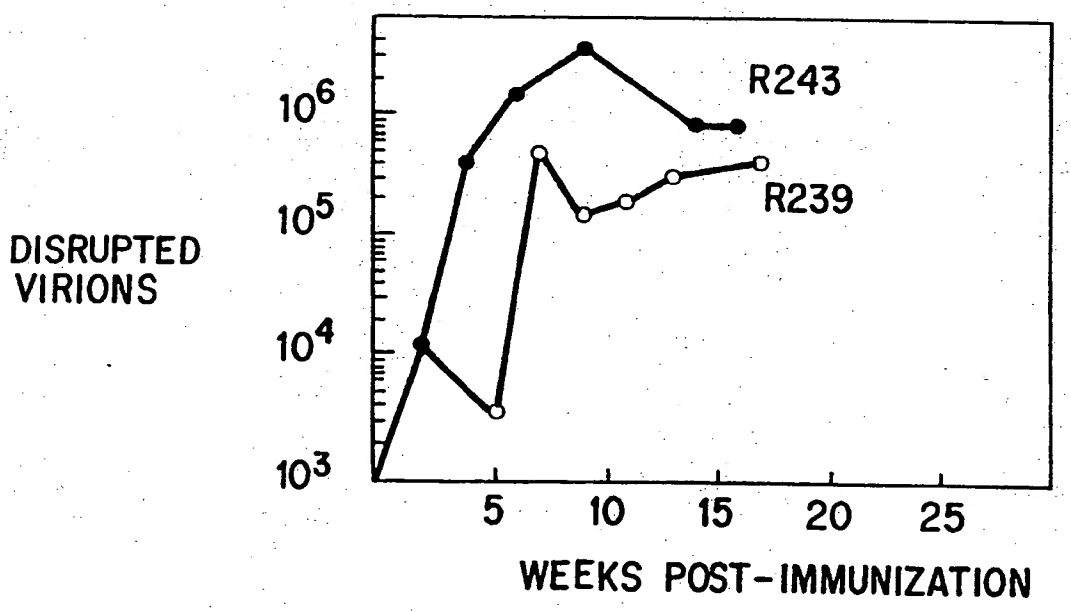


FIG. 15B

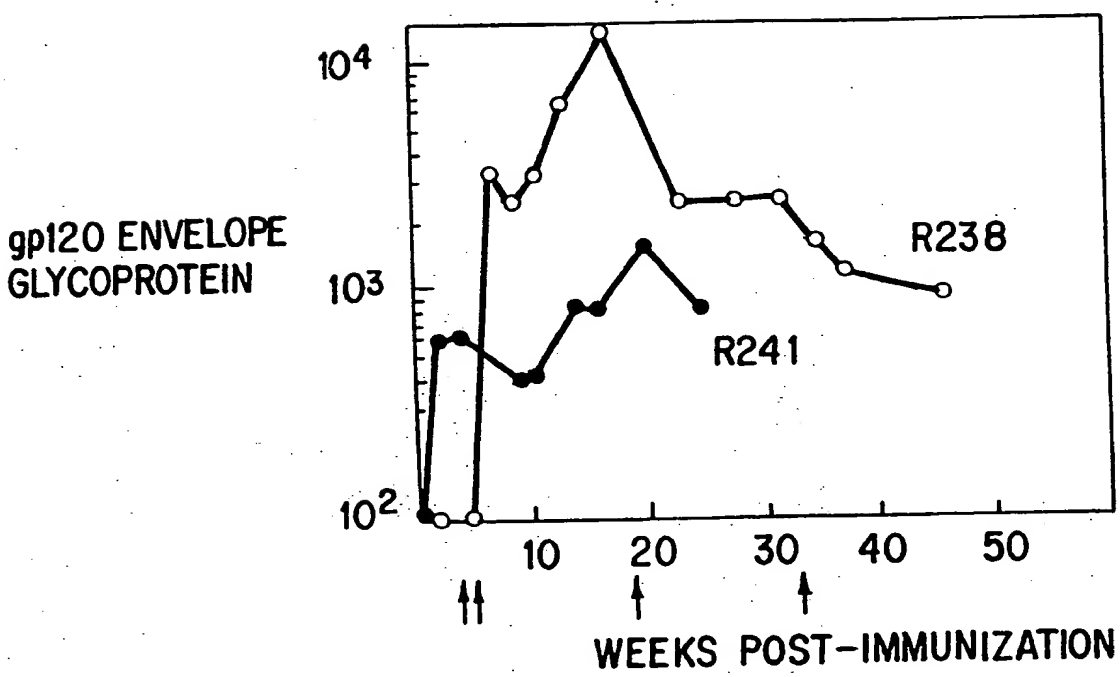


FIG. 15C

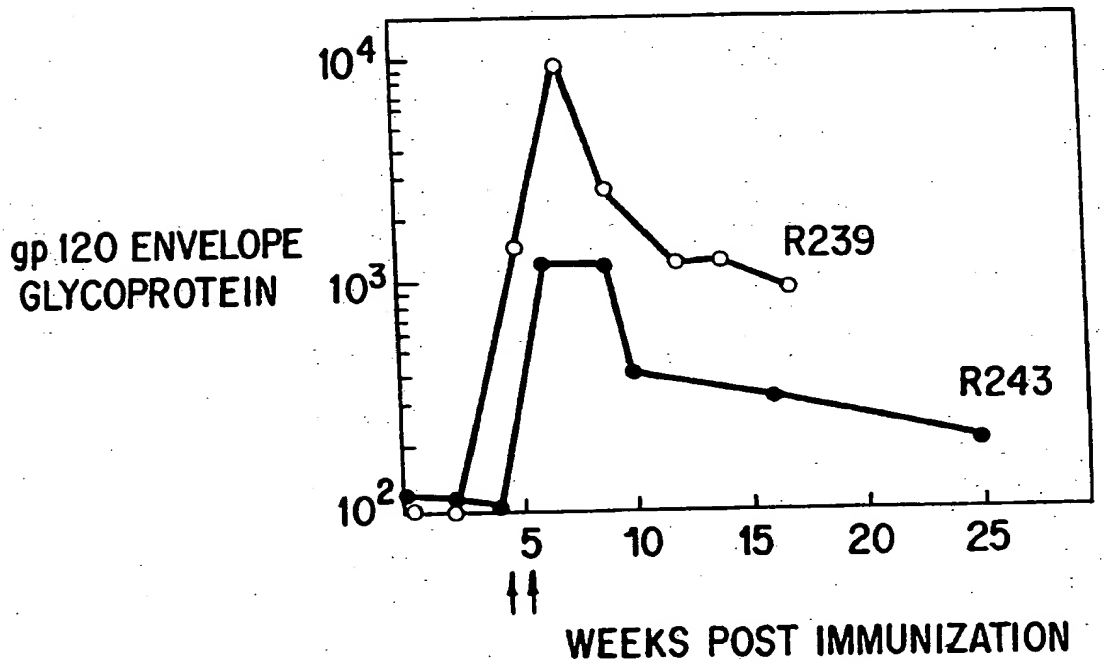


FIG. 15D

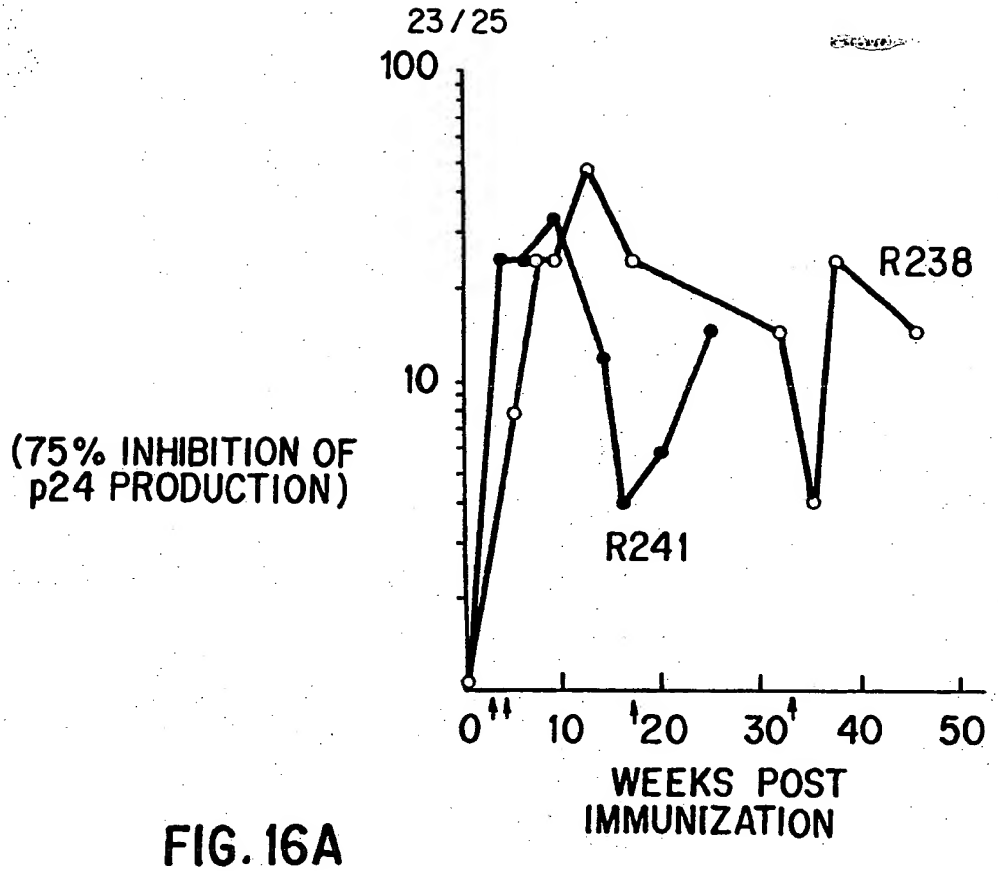


FIG. 16A

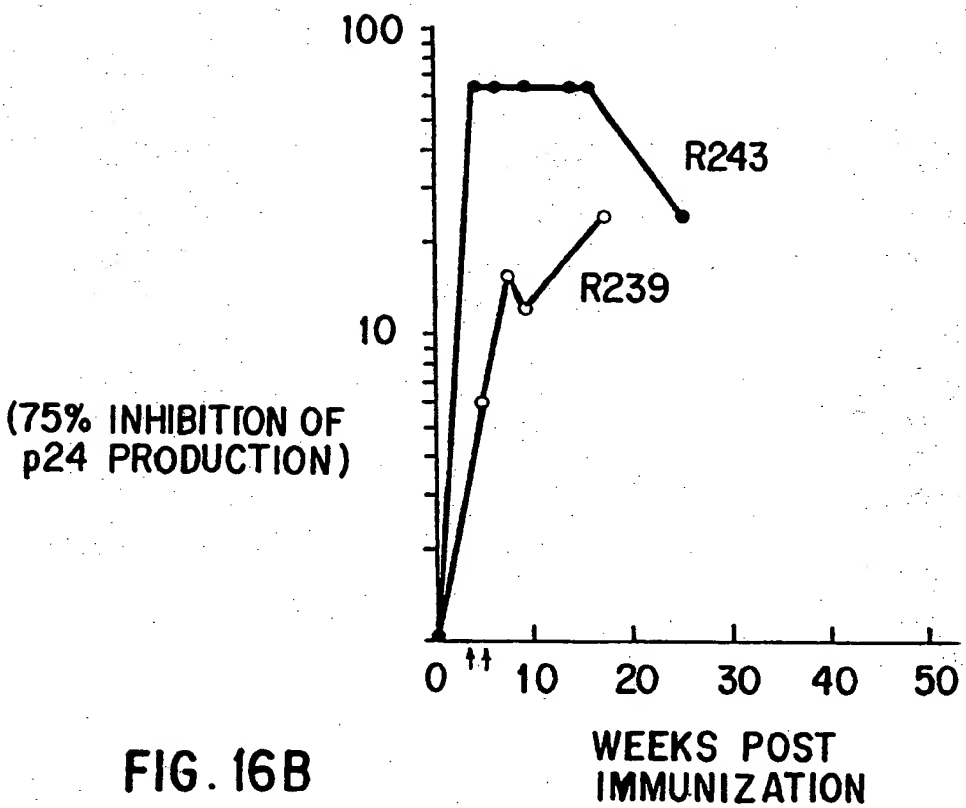
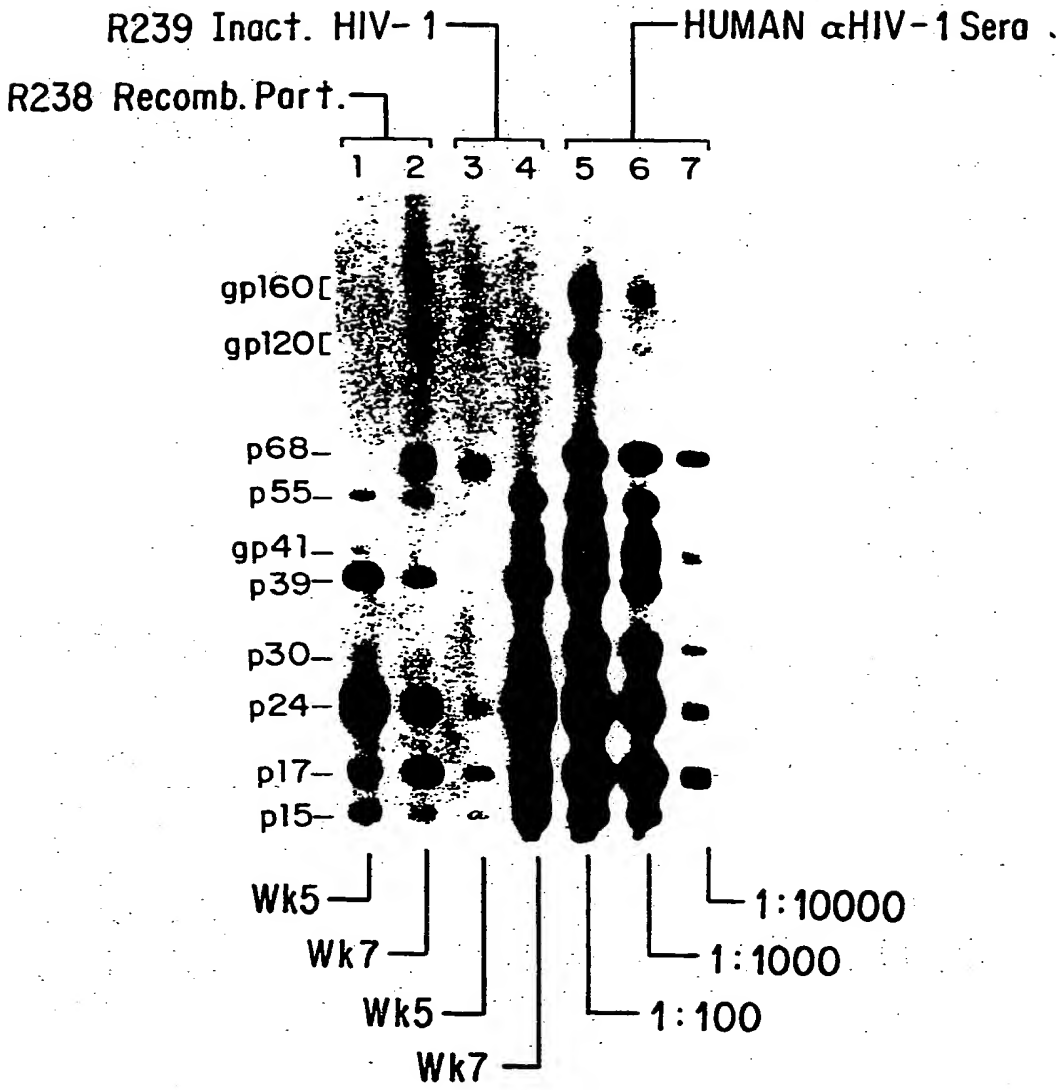


FIG. 16B

FIG. 17



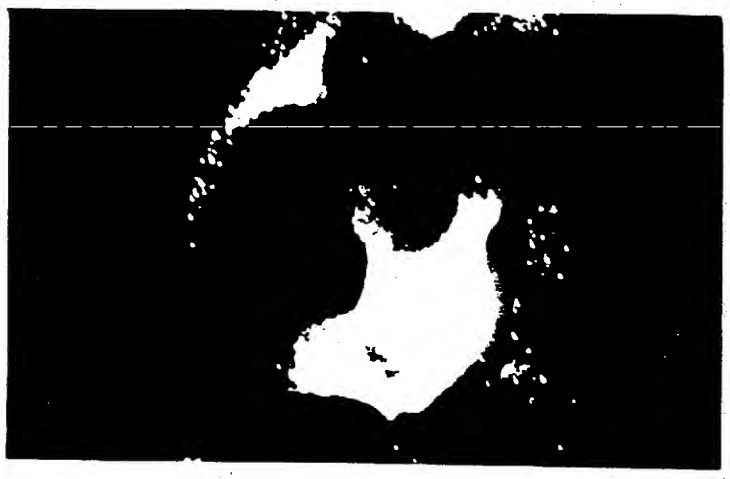


FIG. 18A

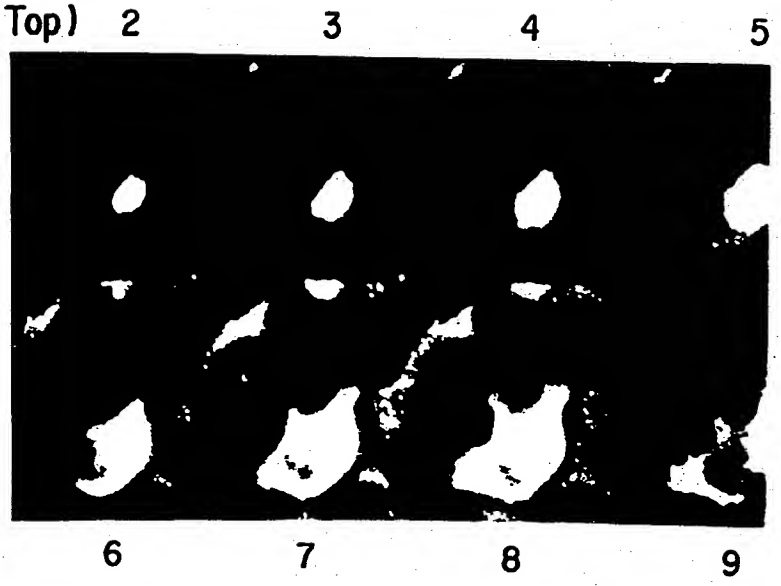


FIG. 18B

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US90/06798

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5):C07K 1/00, 15/04

U.S. CL.: 536/27; 435/69.1

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	536/27; 435/69.1

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

Databases:Dialog (files 157, 155, 154, 399, 5, 72, 172) USPTO Automated Patent System (File USPAT 1971-1990)

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
A	Virology. volume 156. issued 1987. Gelderblom et al.. "Fine Structure of Human Immunodeficiency Virus (HIV) and Immunolocalization of Structural Proteins". pages 171-176.	1.18.22. 35-39.76
Y	Science. volume 240. issued 10 June 1988. Kong et al.. "West African HIV-2-Related Human Retrovirus with Attenuated Cytopathicity" pages 1525-1529. see entire document.	1.18.22. 35-39.76
Y,P	Journal of Virology. volume 64. no. 7. issued July 1990. Gorelick. et al. "Noninfectious Human Immunodeficiency Virus Type 1 Mutants Deficient in Genomic RNA", pages 3207-3211. see pages 3207-3211. see page 3207-3208.	1.18.22, 35-39,76

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

14 February 1991

Date of Mailing of this International Search Report

15 MAR 1991

International Searching Authority

ISA/US

Signature of Authorized Officer

Lynette F. Smith

Lynette F. Smith

ebw

Form PCT/ISA/210 (second sheet) (Rev.11-87)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . because they relate to subject matter [2] not required to be searched by this Authority, namely:

2. ☐ Claim numbers . because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [3], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

See attached sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers: 1,18,22, 35-39, 76

Telephone Practice

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Nature. Volume 336. issued 01 December 1988. Nixon et al. "HIV-1 gag specific cytotoxic T lymphocytes defined with recombinant vaccinia virus and synthetic peptides", pages 484-487. see abstract.	35-39,76
Y	Gene. volume 52. issued 1987. Srinivasan et al.. "Molecular characterization of human immunodeficiency virus from Zaire: nucleotide sequence analysis identifies conserved and variable domains in the envelope gene", pages 71-82 see page 71-73.	1,18,22 35-39,76

Attachment To PCT/ ISA/210

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1, 18, 22, 35-37, 38, 39, 76, drawn to Recombinant HIV particle comprising core and envelope proteins method of making the particle and method of using the particle.

Additional groups are as follows:

group 2 claims 2, 3, 4, 44
group 3 claims 5-8, 42, 9, 80, 81, 10, 78, 11 (requirement for species election of 9 and 80, 81 or 10 and 78 or 11)
group 4, claims 12, 45
group 6, claim 14
group 7, claims 15, 75
group 8, claim 16
group 9, claim 17
group 10, claim 19
group 11, claim 20
group 12, claim 21
group 13, claims 23, 31
group 14, claim 24
group 15, claim 25
group 16, claims 26, 33
group 17, claim 27
group 18, claim 28
group 19, claim 29
group 20, claim 30
group 21, claim 32
group 22, claim 34
group 23, claim 41 (if this group is elected, then a further election of specie in the Markush group is required).
group 24, claim 46
group 25, claim 47
group 26, claim 40
group 27, claim 48
group 28, claim 49 (if this group is elected, then an ultimate specie election is required)
group 29, claim 50
group 30, claim 51
group 31, claim 52
group 32, claim 53
group 33, claim 54
group 34, claim 55
group 35, claim 56
group 36, claim 57 (if this group is elected, then a requirement for election of ultimate specie is requested.)

Attachment To PCT/ ISA/210
Continued

group 37, claim 58, (If this group is elected, requirement for election of ultimate specie).
group 38, claim 59
group 39, claim 60
group 40, claim 61
group 41, claim 62
group 42, claim 63
group 43, claim 64
group 44, claim 65
group 45, claim 66 (if this group is elected, then a further election of species is required in the Markush group),
group 46, claim 67
group 47, claim 68
group 48, claim 69
group 49, claim 70
group 50, claim 71
group 51, claim 72
group 52, claim 73
group 53, claim 74
group 54, claim 77
group 55, claim 79
group 56, claim 82
group 57, claim 83
group 58, claim 84
group 59, claim 85
group 60, claim 86

The inventions listed as Groups 2-60 do not meet the requirements for Unity of Invention for the following reasons: the methods use different genes coding for different viral proteins. The products contain differed viral proteins. The products and methods of making the products and methods of using the products were grouped together as much as possible. Each specie , beyond the first of any group, elected must be paid for at $150 per specie.

Any inquiry concerning this communication should be directed to Lynette F. Smith at telephone number 703-308-0376.